消化管運動ペースメーカーの細胞組織学的研究

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6、研究発表

ア、学会誌等

Komuro T. and Seki K. Fine structural study of interstitial cells associated with the deep muscular plexus of the rat small intestine, with special reference to the intestinal pacemaker cells. Cell Tissue Res. 282: 129-134, 1995

Zhou D.S. and Komuro T. Ultrastructure of the zinc iodide-osmic acid stained cells in guinea pig small intestine. J. Anat. 187: 481-485, 1995

Komuro T. and Zhou D.S. Anti-c-*kit* protein immunoreactive cells corresponding to the interstitial cells of Cajal in the guinea-pig small intestine. J. Auton. Nerv. Syst. 61: 169-174, 1996

Komuro T., Tokui K. and Zhou D.S. Identification of the interstitial cells of Cajal. Histol. Histopathol. 11: 769-786, 1996

Ishikawa K. and Komuro T. Characterization of the interstitial cells associated with the sub-muscular plexus of the guinea-pig colon. Anat. Embryol. 194: 49-55, 1996

Ishikawa K., Komuro T., Hirota S. and Kitamura Y. Ultrastructural identification of the c-*kit*-expressing interstitial cells in the rat stomach, using Ws /Ws mutant rats. Cell Tissue Res. 280: 97-111, 1997

Seki K. and Komuro T. Further observation of the gap junction-rich cells in the deep muscular plexus of the rat small intestine. Anat. Embryol. 197: 97-111, 1998

Horiguchi K., and Komuro T. Ultrastructural characterization of interstitial cells of Cajal in the rat small intestine using control and Ws /Ws mutant rats. Cell Tissue Res 293: 277-284, 1998

イ、口頭発表

堀口和秀　周徳山　関馨介　広田誠一　北村幸彦　小室輝昌：Cajal の介在細胞の c-*kit* 遺伝子発現に関する形態学的検討、日本平滑筋学会（1995）

周徳山　小室輝昌：Cajal の介在細胞の細胞学的特性、第１０１回日本解剖学会（1996）

堀口和秀　周徳山　北村幸彦　小室輝昌：c-*kit* 欠損ラット（Ws/Ws）による消化管ペースメーカー細胞の解、第１０１回日本解剖学会（1996）

関馨介　小室輝昌：超薄切片立体再構築による ICC-DMP の解析、日本平滑筋学会（1996）

石川浩一　小室輝昌：ラット胃輪走筋内 c-*kit* 発現間質性細胞の微細構造、日本電子顕微鏡学会（1997）

関馨介　小室輝昌：モルモット消化管 c-*kit* 発現細胞の免疫組織化学的研究、　日本解剖学会関東地方会（1997）

堀口和秀　小室輝昌：ラット小腸 c-*kit* 発現細胞の微細構造、日本解剖学会関東地方会（1997）

Komuro T, Zhou DS, Seki K： Cytological identification of the interstitial cells of Cajal. 日欧米消化管運動シンポジウム　Three decades of advances in gastrointestinal motility. (1997)

Seki K, Zhou DS, Komuro T： Distribution of anti-c-*kit* protein immunoreactive cells and connexin 43 in the guinea-pig digestive tract.　日欧米消化管運動シンポジウム　Three decades of advances in gastrointestinal motility. (1997)

Komuro T, Ishikawa K, Horiguchi K： Morphological characterization of the interstitial cells of Cajal. Inaugural Congress of International Society for Autonomic Neuroscience. (1997)

7、研究成果

ア、研究の背景および目的：

消化管平滑筋によるぜんどう運動は、消化、吸収、排泄を正常に維持する上で重要な機能であるが、その自動能を発現するペースメーカーの所在については未解決の状態にある。

消化管の制御機構に関して、申請者は筋層間神経叢の構築、神経終末型等の研究から、当該分野に於ける細胞学上の歴史的論争課題である Interstitial cells of Cajal(ICC) の研究へと進んできたが、同時期に提出された同細胞の消化管運動ペースメーカーとしての仮説（Thuneberg 1982）に触れ、その後の研究を継続してきている。

本研究では、細胞全体の形状、細胞骨格、微細構造、周辺組織との関係等の精細、包括的な検討により ICC の細胞学的起源を明かにすると共に、消化管運動調節機構における役割について解析する。

最近のICC の研究については、ヒトの手術材料による形態学的研究、培養系に移した細胞での生理学的研究なども散見される様になったが、根本的な細胞学的同定に未だ研究者間の一致が見られない状態にあり、今後の多方面に渡る研究の進展の為にも、研究の土台部分に当たる細胞組織像の充分な解析が急務と考えられる。

本研究によって得られた成果は、尿管等、同じくペースメーカーの主張されている臓器に於ける研究進展にも寄与するであろう。また、ICC は、交感神経系の末梢部で刺激を効果器に伝達する装置として Cajal(1911) によって示唆されたものであり、交感神経系終末部に於ける歴史的問題に明確な解答を与えることになろう。

イ、研究実施計画と結果：

＊平成７年度には、ペースメーカーとして提唱されているカハールの介在細胞（Interstitial Cells of Cajal; ICC ）の細胞学的性格を明らかにするため、古典的染色法との共通性を持つヨウ化アエンーオスミウム酸法（ZI0）を用い、観察をおこなった。①その結果、モルモット小腸筋層間神経叢の部位には、神経線維網とは別に、細胞性網状構造を構成する突起を持った多数の細胞を確認した。細胞の形、大きさ、突起の分枝の形状等から、これらのZI0陽性細胞はCaja1の原著のICCに相当すると結論した。②同試料のエポキシ樹脂包埋切片による電子頭微鏡的検索では、これらの細胞は線維芽細胞と似た特徴を有し、神経細胞、平滑筋細胞とは異なることが明らかとなった。

＊平成８年度には、細胞の発生学的由来を検証するため、ペースメーカーとの密接な関係が示唆されているc-kit receptorの発現の有無、細胞骨格蛋白等について免疫組織化学的に調べた。③その結果、モルモット小腸筋層間神経叢には、抗c-Kit 抗体（ACK2)、抗ヴィメンチン抗体免疫染色およびZIO 染色のいずれにおいても、同一の特徴を有する長い突起を持った多数の細胞が観察された。これらの成果から、この部位の ICC は*c-kit* 発現細胞に一致するものであり、ペースメーカー機能を持つものと推定した。また、細胞骨格としてヴィメンチンを多量に含むことから、同細胞は間葉系に由来するものと結論した

＊平成９年度には、c-kit 遺伝子に突然変異をもつ Ws/Ws ラットおよび同腹の正常ラット +/+ を用い、消化管各部位におけるc-kit 発現細胞の細胞学的検索を通して、ペースメーカー細胞の特性、ICC の異型性等について検討した。その結果、④筋層間神経叢のICCは、消化管の部位（胃、小腸、大腸）に拘わらず、共通の微細構造（豊富なミトコンドリア、大きな gap-junction の形成など）よって特徴ずけられることを確認した。また、これらの細胞は Ws/Ws ラットでは観察されなかった。⑤一方、小腸の深部筋神経叢及び大腸の筋層下神経叢のICCでは、上記の特徴に加え、基底膜、caveolae を 示したが、この細胞は Ws/Ws ラットでも観察された。以上の観察結果から、c-kit 発現細胞あるいはICCは細胞学的に不均一な細胞群からなり、ペースメーカーとしては筋層間神経叢のICCが第一義的な役割を持つ一方、その他の部位のICCについては、一層詳細な検索、考察が必要なものと結論した。

8、発表論文

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Short Report

Ultrastructure of the zinc iodide–osmic acid stained cells in guinea pig small intestine

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ABSTRACT

This study has demonstrated that cells stained using the zinc iodide–osmic acid (ZIO) method have the same fine structural features as those of fibroblasts. They have a well developed Golgi apparatus, granular endoplasmic reticulum and many mitochondria. They have no basal lamina. They are distributed in association with the deep muscular plexus, within the outer circular muscle layer and in the space between the circular and longitudinal muscle layers. Since this staining method is believed to co-stain nerves and interstitial cells of Cajal, we concluded that these ZIO-positive, fibroblast-like cells represent at least some, if not all, of the interstitial cells which appeared in the original description by Cajal.

Key words: Interstitial cells of Cajal; fibroblasts; intestine; guinea pig; zinc iodide–osmic acid stain.

INTRODUCTION

Although recent studies have provided evidence to support a modern hypothesis (Thuneberg, 1982, 1989) that interstitial cells of Cajal (ICC) display pace-making activity in gut movement (Hara et al. 1986; Suzuki et al. 1986; Huizinga et al. 1988; Du & Conklin, 1989; Serio et al. 1991; Liu et al. 1994), the nature of ICC remains essentially unresolved since Cajal described 'cellule nerveuse interstitielle', implying that they represent primitive sympathetic neurons based on their staining affinity by the methylene blue and Golgi methods (Cajal, 1893, 1911).

Different types of interstitial cells have been distinguished ultrastructurally in the deep muscular plexus or myenteric plexus at different levels of the alimentary tract in a numer of animals, including the rabbit (Komuro, 1982), mouse (Rumessen et al. 1982), rat (Komuro, 1989), guinea pig (Zhou & Komuro, 1992*a*, *b*) and dog (Berezin et al. 1988; Torihashi et al. 1993), as well as in man (Rumessen et al. 1992, 1993).

It has been suggested that the zinc iodide–osmic acid (ZIO) method (Maillet, 1959) has staining properties similar to those of methylene blue and silver impregnation, and that it co-stains nerves and ICC (Taxi, 1965; Rumessen et al. 1982). Because of the high content of osmium tetroxide in the fixative-staining solution, the ZIO method provides both good preservation and high electron density. It therefore offers the advantage of the ability to examine the same specimens by both light and electron microscopy.

The purpose of the present study was to clarify the ultrastructural features of the ZIO-positive cells in guinea pig small intestine in order to elucidate the nature of ICC.

MATERIALS AND METHODS

Young adult guinea pigs (aged 4 wk) of both sexes were used. Under ether anaesthesia, short segments of jejunum were removed and immediately placed in Tyrode's solution containing 1 mM papaveline to avoid nonspecific staining of the smooth muscle cells (Rumessen & Thuneberg, 1982). Then the specimens were moderately inflated with Tyrode's solution and immersed in a freshly prepared ZIO mixture composed of 0.4% OsO_4 and 2.4% ZnI_2 for 10–24 h. After rinsing in several changes of distilled water, the specimens were carefully laminated under a dissecting microscope to make whole mount preparations of the circular muscle layer containing the deep muscular

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plexus and/or the longitudinal muscle layer attached to the myenteric plexus. The specimens were observed and photographed by a Nikon photomicroscope, and were subsequently cut into small pieces (~ 2–3 mm) and processed for electron microscopic examination. The specimens were block-stained with 3% uranyl acetate solution, dehydrated in a graded series of ethyl alcohols and embedded in epoxy resin. Thin sections were cut and double-stained with 3% uranyl acetate followed by lead tartrate, and were examined using a Hitachi HU-12A electron microscope. The specimens of the jejunum were also processed for electron microscopy without ZIO staining.

RESULTS

In whole mount preparations, the deep muscular plexus is observed as a 2-dimensional nerve network composed of rectangular or polygonal arrays extending between the inner and outer sublayers of the circular muscle. Many interstitial cells stained dark grey are closely associated with the deep black varicose axons (Fig. 1). They have oval nuclei and show similar cell axes to those of the circular muscle cells. They are generally bipolar or stellate in shape and their cytoplasmic processes extend for considerable distances along the nerve bundles.

Cells with similar features surrounding the nerve bundles within the outer main layer of the circular muscle are also stained by the ZIO method (Fig. 2). Their cell processes are often more clearly observed in this location than in the deep muscular plexus because of fewer instances of their superimposition on the less dense nerve plexus. However, the ZIO-positive cells in the region of the myenteric plexus region are observed in the interstices of the tertiary plexus, and they appear to form an independent cellular network (Fig. 3).

On electron microscopy, the ZIO-positive cells are clearly identified by the high electron density caused by precipitation of the ZnI_2 and OsO_4 mixture which fills their entire cytoplasm (Fig. 4). In longitudinal sections of the intestine (perpendicular to the main axis of the deep muscular plexus), ZIO-positive cells

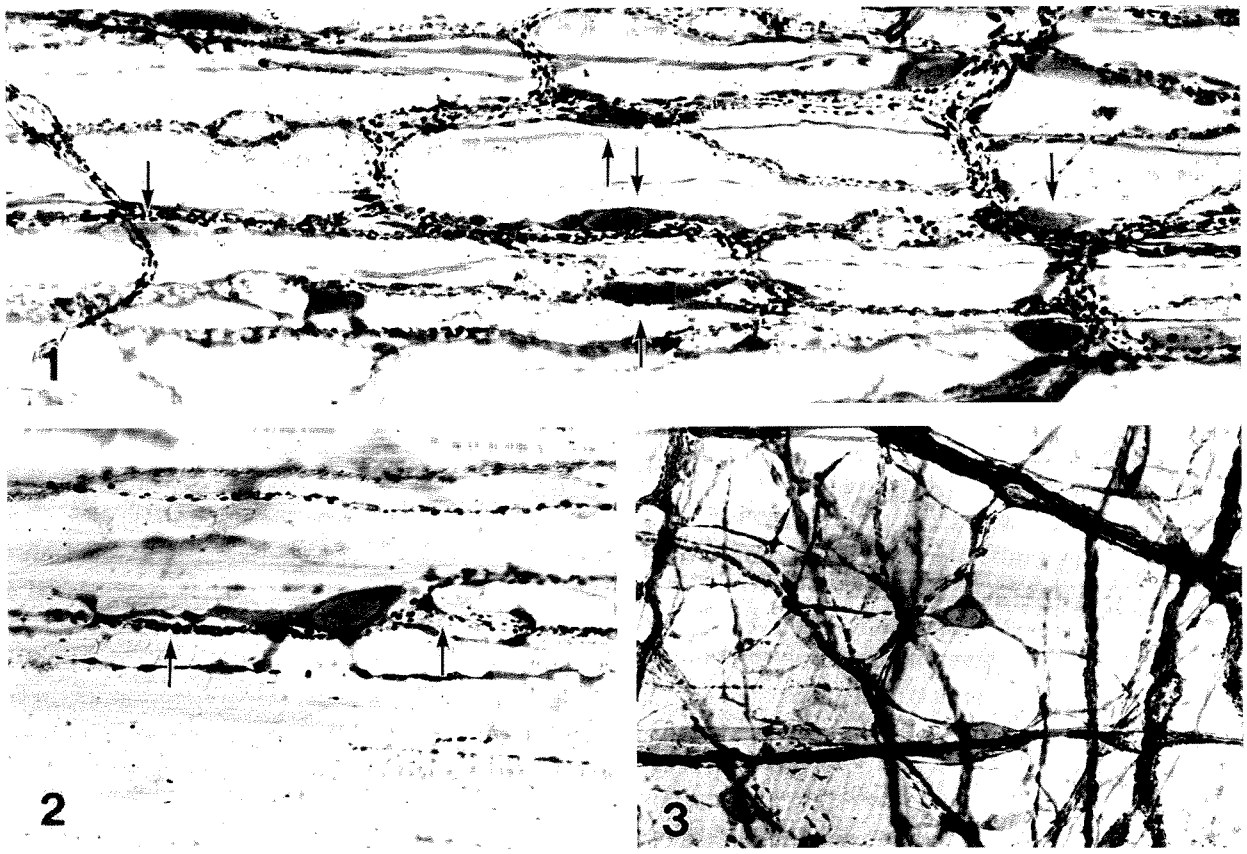


Fig. 1. Whole mount preparation of the guinea pig small intestine showing the deep muscular plexus associated with darker grey interstitial cells (arrows). The axis of the nerve network is parallel to that of the circular muscle cells. ZIO staining, × 125.

Fig. 2. Whole mount preparation showing a ZIO-positive cell within the circular muscle layer. The cell body is closely associated with the varicose nerve bundle (arrows). ZIO staining, × 270.

Fig. 3. Whole mount preparation showing ZIO-positive cells located in the interstices of the secondary and tertiary nerve network of the myenteric plexus. They have 2–5 processes extending in all directions. ZIO staining, × 125.

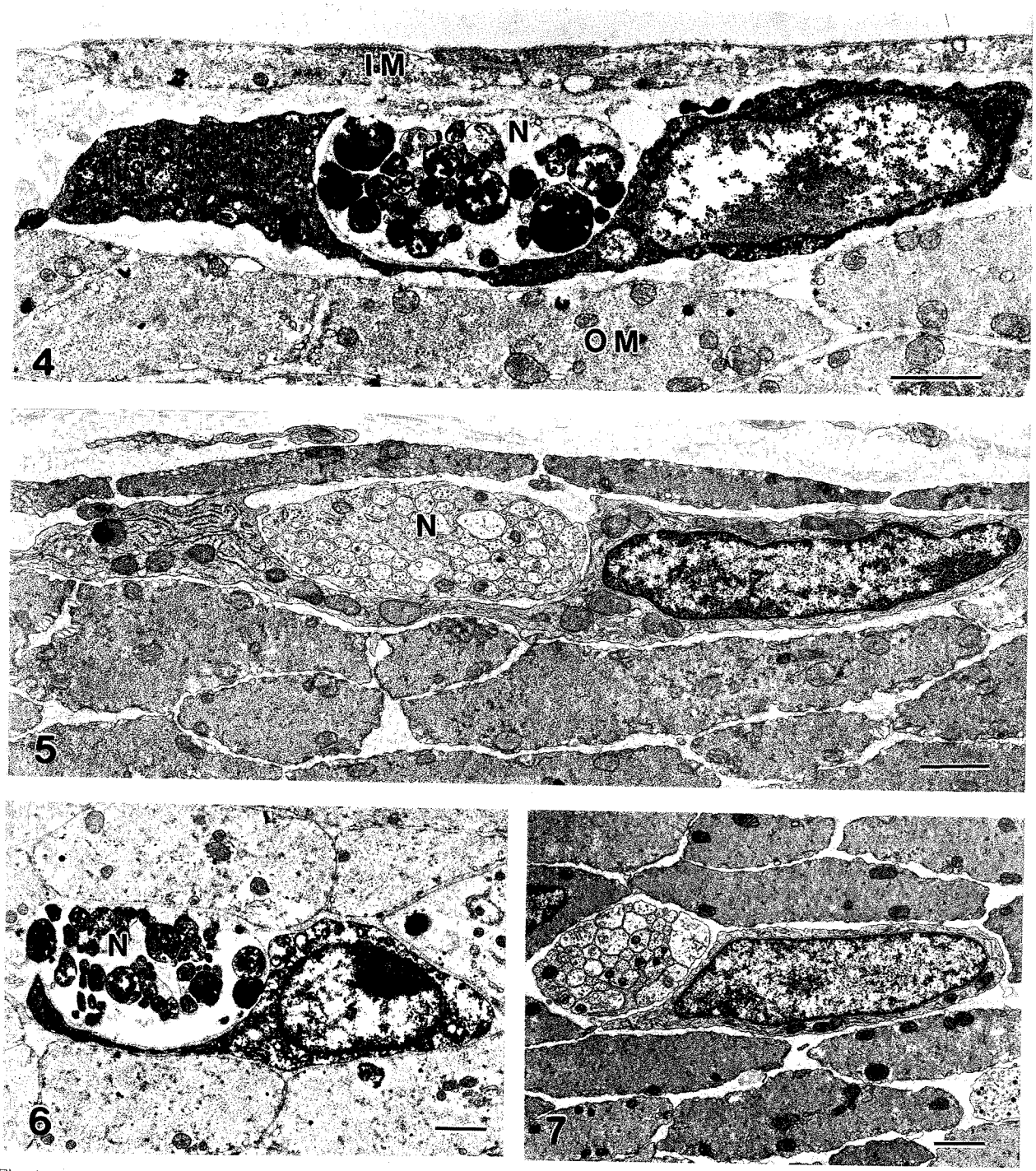


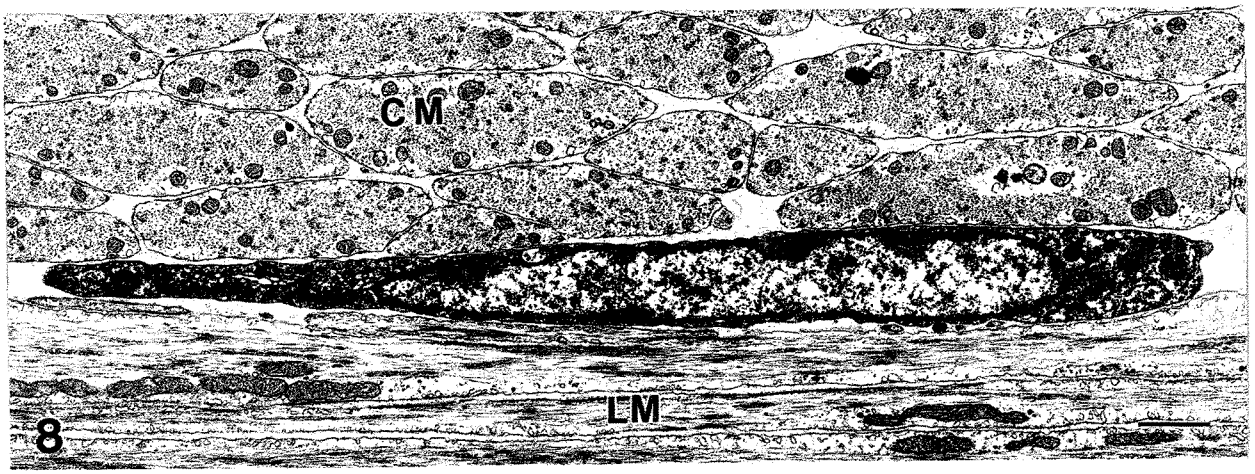
Fig. 4. Electron micrograph showing a ZIO-positive cell associated with the deep muscular plexus. Its process envelops the deeply stained nerve bundle (N) as an incomplete sheath. IM and OM represent the inner and outer layers of the circular muscle, respectively. Bar, 1 μm.

Fig. 5. Fibroblast-like cell associated with a nerve bundle (N) of DMP. Many mitochondria and a well developed granular endoplasmic reticulum are observed in the cytoplasm. Bar, 1 μm.

Fig. 6. A ZIO-positive cell partly envelops a nerve bundle (N) within the circular muscle layer. Bar, 1 μm.

Fig. 7. A fibroblast-like cell located beside a nerve bundle within the circular muscle layer. Bar, 1 μm.

are observed to envelop the nerve bundles as an incomplete sheath, where nerve fibres are usually co-stained with a higher density. This electron-dense precipitation often obscures the cytoplasmic features of the ZIO-positive cells, so that the localisation of membrane-bound cell organelles such as mitochondria and endoplasmic reticulum can hardly be identified. Moderately stained preparations help to identify their equivalents. In the specimens not treated with ZIO, the cells are characterised by fibroblastic



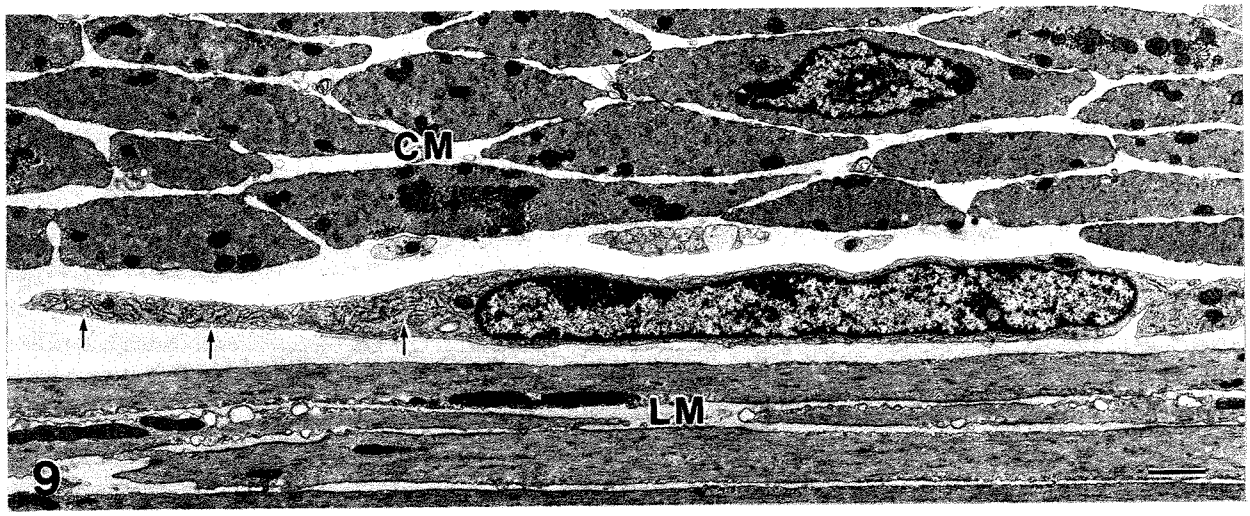


Fig. 8. A ZIO-positive cell between the circular (CM) and longitudinal (LM) muscle layers. Many mitochondria, GER and Golgi apparatus can be distinguished in the perinuclear cytoplasm. Bar, 1 μm.

Fig. 9. A fibroblast-like cell located between the circular (CM) and longitudinal (LM) muscle layers. Well-developed granular endoplasmic reticulum is seen in the cytoplasm (arrows). Bar, 1 μm.

features, i.e. well developed granular endoplasmic reticulum, Golgi apparatus and many mitochondria scattered throughout the cytoplasm. They have no basal lamina (Fig. 5).

Within the outer circular muscle layer, ZIO-positive cells are observed in close proximity to the nerve bundles (Fig. 6), and correspond to interstitial cells with fibroblastic features (Fig. 7), which are the only type of cell except free cell components in this layer. ZIO-positive cells showing the ultrastructure of fibroblasts are also found around the myenteric ganglia and in spaces between the circular and longitudinal muscle layers (Figs 8, 9). Although the ZIO method occasionally blackens a variable structure, the fibroblastic cells described above were consistently well stained under the controlled conditions of the present study. Schwann cells and free cells such as macrophages were not stained.

DISCUSSION

The present study has demonstrated that a number of ZIO-positive cells distributed in the region of the deep muscular plexus, within the outer circular muscle layer and in the myenteric plexus region, are characterised by the fine structure of fibroblasts.

Provided the ZIO method has the same staining affinity to ICC as methylene blue or silver impregnation (Taxi, 1965; Rumessen & Thuneberg, 1982), ICC depicted by the ZIO method in the guinea pig (Taxi, 1965; Kobayashi et al. 1989), mouse (Rumessen & Thuneberg, 1982) and the cat, dog, ferret, opossum, rat and rabbit (Christensen et al. 1992), probably represent the fibroblast-like cells, although this is not compatible with the observation that ICC-III have more similarities with smooth muscle cells than the former (Rumessen et al. 1982; Faussone-Pellegrini, 1987).

As mentioned above, ultrastructurally different

types of interstitial cells have been reported at different levels of the alimentary tract of some animals, including the rabbit (Komuro, 1982), mouse (Rumessen et al. 1982), rat (Komuro, 1989), guinea pig (Zhou & Komuro, 1992*a*, *b*) and dog (Berezin et al. 1988; Torihashi et al. 1993), as well as in man (Rumessen et al. 1992, 1993). However, it is not certain whether they are all identifiable as the same type of cell or ICC. Moreover, there is also some doubt as to whether the ICC originally described in the myenteric plexus region, in the deep muscular plexus and within the circular muscle layer of the guinea pig and rabbit (Cajal, 1893, 1911) really belong to a single category of cell.

We are aware that none of the methods applied hitherto has been for the specific staining for ICC, as pointed out by Thuneberg (1982). Moreover, we cannot exclude the possibility that the ICC depicted in the original description included heterogeneous populations of cells, for the same reason, or because of a lack of specific staining. Instead, co-staining of ICC and the neuronal components seems to be important when considering the nature of ICC. The present observations clearly demonstrate that the ZIO method stained nerves and fibroblast-like cells simultaneously. It thus can be concluded that the ZIO-positive fibroblast-like cells represent at least some, if not all, of the ICC which appeared in the original description by Cajal (1893, 1911).

REFERENCES

Berezin I, Huizinga JD, Daniel EE (1988) Interstitial cells of Cajal in the canine colon: a special communication network at the inner border of the circular muscle. *Journal of Comparative Neurology* **273**, 42–51.

Cajal SR y (1893) Sur les ganglions et plexus nerveux de l'intestin. *Compte Rendu des Séances de la Société de Biologie, Paris* **45**, 217–223.

Cajal SR y (1911) *Histologie du Système Nerveux de l'Homme et des Vertébrés*, vol. 2. Paris: Maloine.

Christensen J, Rick GA, Lowe LS (1992) Distributions of interstitial cells of Cajal in stomach and colon of cat, dog, ferret, opossum, rat, guinea-pig and rabbit. *Journal of the Autonomic Nervous System* **37**, 47–56.

Du C, Conklin JL (1989) Origin of slow waves in the isolated proximal colon of the cat. *Journal of the Autonomic Nervous System* **28**, 167–178.

Faussone-Pellegrini MS (1987) Comparative study of interstitial cells of Cajal. *Acta Anatomica* **130**, 109–126.

Hara Y, Kubota M, Szurszewski JH (1986) Electrophysiology of smooth muscle of the small intestine of some mammals. *Journal of Physiology (London)* **372**, 501–520.

Huizinga JD, Shin A, Chow E (1988) Electrical coupling and pacemaker activity in colonic smooth muscle. *American Journal of Physiology* **255**, C653–C660.

Kobayashi S, Furness JB, Smith TK, Pompolo S (1989) Histological identification of the interstitial cells of Cajal in the guinea-pig small intestine. *Archives of Histology and Cytology* **52**, 267–286.

Komuro T (1982) The interstitial cells in the colon of the rabbit: scanning and transmission electron microscopy. *Cell and Tissue Research* **222**, 41–51.

Komuro T (1989) Three-dimensional observation of the fibroblast-like cells associated with the rat myenteric plexus, with special reference to the interstitial cells of Cajal. *Cell and Tissue Research* **255**, 343–351.

Liu LWC, Thuneberg L, Huizinga JD (1994) Selective lesioning of interstitial cells of Cajal by methylene blue and light leads to loss of slow waves. *American Journal of Physiology* **266**, G485–G496.

Maillet M (1959) Modifications de la technique de Champy au tétroxyde d'osmium-iodure du potassium. Résultats de son application à l'étude des fibres nerveuses. *Compte Rendu des Séances de la Société de Biologie* **53**, 939–940.

Rumessen JJ, Thuneberg L (1982) Plexus muscularis profundus and associated interstitial cells. I. Light microscopical studies of mouse small intestine. *Anatomical Record* **203**, 115–127.

Rumessen JJ, Thuneberg L, Mikkelsen HB (1982) Plexus muscularis profundus and associated interstitial cells. II. Ultrastructural studies of mouse small intestine. *Anatomical Record* **203**, 129–146.

Rumessen JJ, Mikkelsen HB, Thuneberg L (1992) Ultrastructure of interstitial cells of Cajal associated with deep muscular plexus of human small intestine. *Gastroenterology* **102**, 56–68.

Rumessen JJ, Mikkelsen HB, Qvortrup K, Thuneberg L (1993) Ultrastructure of interstitial cells of Cajal in circular muscle of human small intestine. *Gastroenterology* **104**, 343–350.

Serio R, Barajas-Lopez C, Daniel EE, Berezin I, Huizinga JD (1991) Slow-wave activity in colon: role of network of submucosal interstitial cells of Cajal. *American Journal of Physiology* **260**, G636–G645.

Suzuki N, Prosser CL, Dahms V (1986) Boundary cells between longitudinal and circular layers: essential for electrical slow waves in cat intestine. *American Journal of Physiology* **250**, G287–G294.

Taxi J (1965) Contribution à l'étude des connexions des neurones moteurs du système nerveux autonome. *Annales des Sciences Naturelles, Zoologie (Paris)* **7**, 413–674.

Thuneberg L (1982) Interstitial cells of Cajal: intestinal pacemaker cells? *Advances in Anatomy, Embryology and Cell Biology* **71**, 1–130.

Thuneberg L (1989) Interstitial cells of Cajal. In *Handbook of Physiology. The Gastrointestinal System*, vol. 1 (ed. J. D. Wood), pp. 349–386. Bethesda, MD: American Physiological Society.

Torihashi S, Kobayashi S, Gerthoffer WT, Sanders KM (1993) Interstitial cells in deep muscular plexus of canine small intestine may be specialized smooth muscle cells. *American Journal of Physiology* **265**, G638–G645.

Zhou DS, Komuro T (1992*a*) Interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine, with special reference to the interstitial cells of Cajal. *Cell and Tissue Research* **268**, 205–216.

Zhou DS, Komuro T (1992*b*) The cellular network of interstitial cells associated with deep muscular plexus of the guinea-pig small intestine. *Anatomy and Embryology* **186**, 519–527.

Cell Tissue Res (1995) 282:129–134

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Fine structural study of interstitial cells associated with the deep muscular plexus of the rat small intestine, with special reference to the intestinal pacemaker cells

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Abstract. Two types of interstitial cells have been demonstrated in close association in the deep muscular plexus of rat small intestine, by electron microscopy. Cells of the first type are characterized by a fibroblastic ultrastructure, i.e. a well-developed granular endoplasmic reticulum, Golgi apparatus and absence of the basal lamina. They form a few small gap junctions with the circular muscle cells and show close contact with axon terminals containing many synaptic vesicles. They may play a role in conducting electrical signals in the muscle tissue. Cells of the second type are characterized by many large gap junctions that interconnect with each other and with the circular muscle cells. Their cytoplasm is rich in cell organells, including mitochondria, granular endoplasmic reticulum and Golgi apparatus. They show some resemblance to the smooth muscle cells and have an incomplete basal lamina, caveolae and subsurface cisterns. However, they do not contain an organized contractile apparatus, although many intermediate filaments are present in their processes. They also show close contacts with axon terminals containing synaptic vesicles. These gap-junction-rich cells may be regular components of the intestinal tract and may be involved in the pacemaking activity of intestinal movement.

Key words: Small intestine – Pacemaker – Interstitial cell – Ultrastructure – Rat (Wistar)

Introduction

Thuneberg (1982) has hypothesized that interstitial cells of Cajal (ICC) in the regions of the myenteric plexus and the deep muscular plexus (DMP) are regulatory cells, possibly pacemaker cells of the intestinal musculature. Recent physiological studies have indicated that ICC associated with the submuscular plexus (SMP) of the cat colon (Du and Conklin 1989; Conklin and Du 1990) and the dog colon (Serio et al. 1991; Liu et al. 1993, 1994) play an essential role in the generation of slow waves, thus also indicating pacemaker activity.

Although the ICC of the SMP in the colon and those of the DMP in the small intestine have been regarded as equivalent (Berezin et al. 1988), it is not certain which type of cells of the DMP corresponds to the cells of the SMP, since different types of interstitial cells have been observed in the DMP of various animals (Rumessen and Thuneberg 1982; Rumessen et al. 1982; Zhou and Komuro 1992a, b; Torihashi et al. 1993). Indeed, their identification as ICC from the original description (Cajal 1893, 1911) has not been settled. For example, each of three types of cells found in the DMP region of the guinea-pig intestine (Zhou and Komuro 1992a, b) shows some differences from the so-called ICC-III of the mouse intestine (Rumessen and Thuneberg 1982; Rumessen et al. 1982) and from the cells of the dog colon (Berezin et al. 1988) despite all of the cell types mentioned above forming gap junctions that are believed to be an important feature of pacemaker cells. In the absence of a distinctive marker for ICC, their identification is problematic (Thuneberg 1989; Christensen 1992). More recently, ICC have been studied by new methods including reduced nicotineamide-adenine-dinucleotide diaphorase histochemistry (Xue et al. 1993), cholera-toxin subunit-b labeling (Anderson and Edwards 1993), cyclic GMP immunoreactivity (Shuttleworth et al. 1993; Young et al. 1993) and nitric oxide synthase immunoreactivity (Xue et al. 1994). However none of these methods has been fully convincing in the search for specific markers for ICC.

It seems reasonable to expect the existence of a specific type of cell that is characterized by common morphological features among different species, if pacemaker cells truly exist at this location. Thus, it may be practical to approach the question of pacemaker cells apart from the cytological definition of ICC, since it has been also questioned whether ICC are a single population of cells (Komuro et al. 1991).

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From this point of view, we have observed interstitial cells associated with the rat DMP and compare them with those of other laboratory animals, including the mouse (Rumessen and Thuneberg 1982; Rumessen et al. 1982) and guinea-pig (Zhou and Komuro 1992a, b), both of which have been thoroughly investigated. This approach may enable the identification of common ultrastructural features of putative pacemaker cells for different species.

Materials and methods

Pieces of proximal jejunum from the adult rats (Wistar; n=20) were placed in Karnovsky's fixative containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h at 4%. The specimens were rinsed in the same buffer and post-fixed in 1% osmium tetroxide for 2 h at 4°C. The specimens were then rinsed in distilled water, block-stained with saturated uranyl acetate solution for 3 h, dehydrated in a graded series of ethyl alcohols and embedded in Epoxy resin. Ultrathin sections were cut using a Reichert microtome and double-stained with uranyl acetate and lead tartrate for observation under a JEM 1200 EX II electron microscope.

Results

The DMP of the small intestine of rat consists of nerve bundles running parallel to the circular muscle fibers and transverse interconnecting bundles. The plexus extends two-dimensionally in a plane between inner thin (1–3 cells thick) and outer main layers of the circular muscle. These nerve bundles not only are supported by Schwann cells (Fig. 1), as are those of other peripheral nerves, but are also closely associated with two other types of cell.

Cells of the first type are elongated and show the same cell axis as those of the circular muscle cells (Fig. 2). They show cytoplasmic features similar to those of the fibroblasts. Well-developed Golgi apparatus are located in the paranuclear region. Granular endoplasmic reticulum (GER) is observed throughout the cytoplasm and usually contains moderately dense material in dilated cisterns (Fig. 2). Many mitochondria are scattered in the cytoplasm, and lysosomes and multivesicular bodies can be seen. Caveolae are rarely observed along the cell membrane. There is no basal lamina. Unlike typical fibroblasts, they form a few small gap junctions with the muscle of the main layer (Fig. 2, inset). They also show close contact with nerve varicosities containing many synaptic vesicles (Fig. 2).

Cells of the second type are also elongated cells running parallel to the circular muscles (Fig. 3). The nuclei are oval and have smooth contours. Heterochromatin is generally distributed at the periphery. Their contours seldom show deep indentations along the longer axis, unlike those of the neighbouring smooth muscle cells. Their cytoplasm is usually less electron-dense than that of the smooth muscle cells.

The most conspicuous feature of this type of cell is the frequent occurrence of large gap junctions that interconnect the same type of cells and connect with smooth muscle cells of the main circular layer (Figs. 3–6, 8). These gap junctions are generally large and measure up to 1 μm in thin sections.

Well-developed Golgi apparatus are usually located in the paranuclear region (Fig. 5). Mitochondria, GER and free ribosomes are richly distributed throughout the cytoplasm (Fig. 6). Lysosomes and multivesicular bodies are also present (Figs. 3, 8). Microtubules, intermediate filaments and thin filaments run mainly along the longer cell axis but myosin filaments have not been observed. A small number of caveolae are located along the cell membranes, and subsurface cisterns of smooth endoplasmic reticulum can be seen immediately beneath the cell membranes (Fig. 6). An incomplete basal lamina lies around the cell boundaries (Figs. 6, 7). Cilia and basal bodies are occasionally found.

These cells show close contacts with vesiculated varicosities that predominantly contain clear vesicles (Fig. 7). A rare but significant observation is that these cells form gap junctions with muscle cells of the inner sublayer (Fig. 8). This type of cell forms intermediate junctions, but not gap junctions, with the first type of cell.

Cells containing abundant glycogen granules similar to those of guinea-pigs (Zhou and Komuro 1992a, b) have not been identified. However, it is worth noting that some variations exist among the cells forming large gap junctions, with respect to the electron density of the cytoplasm and of cell organells, and particularly to the number of mitochondria.

Discussion

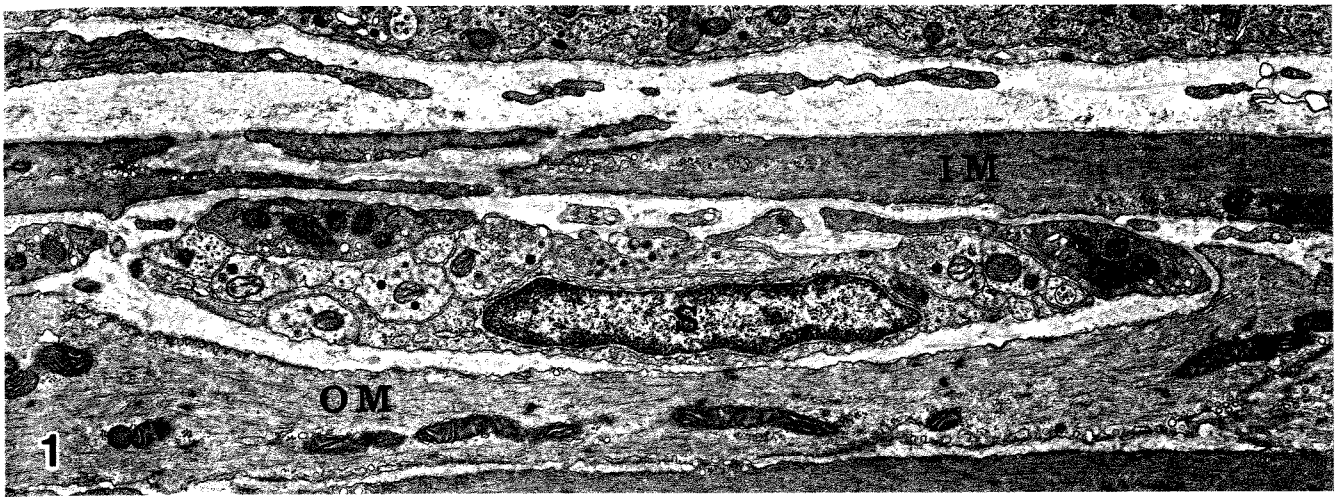
The present study demonstrates that the nerve bundles of the rat DMP are closely associated with two types of interstitial cell. Fibroblast-like cells showing small gap junctions with smooth muscle cells resemble those found in the myenteric region of the rabbit colon (Komuro 1982), the rat small intestine (Komuro 1989) and those of the guinea-pig DMP (Zhou and Komuro 1992a,b). The fibroblast-like cells of the rat intestine are

Fig. 1. Electron micrograph showing a Schwann cell (*S*) of the DMP located between the inner (*IM*) and the outer (*OM*) sublayers of the circular muscle. A part of the submucosal ganglion is seen at the *upper edge* of the micrograph. ×13000

Fig. 2. A fibroblast-like cell (*FL*) observed in close association with the DMP. Dilated cisterns of GER (*ER*) contain moderate electron-dense material. A Golgi apparatus (*G*) is located in the paranuclear region. An axon profile containing clear round vesicles (*N*) is lodged in the surface indentation. ×12500. *Inset* A small gap juction between the fibroblast-like cell and a muscle cell. ×64000

Fig. 3. A gap-junction-rich cell running parallel to the circular muscle (*CM*). The gap junctions with a neighbouring muscle cell are indicated by *arrows*. Multivesicular bodies are seen in the paranuclear region. *SM* Submucosa. ×7000

Fig. 4. A large gap junction between two gap-junction-rich cells. ×140000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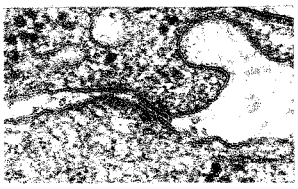

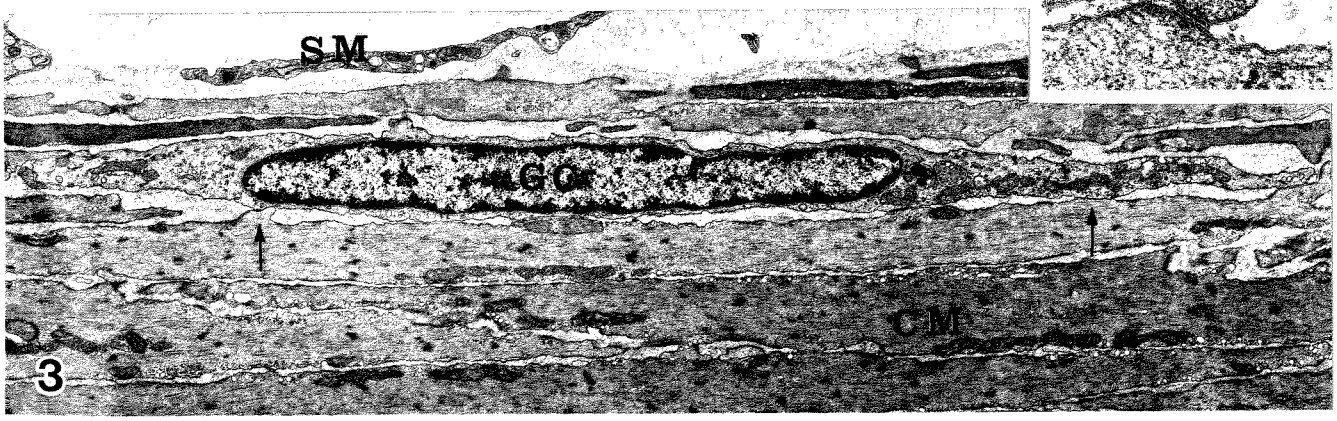
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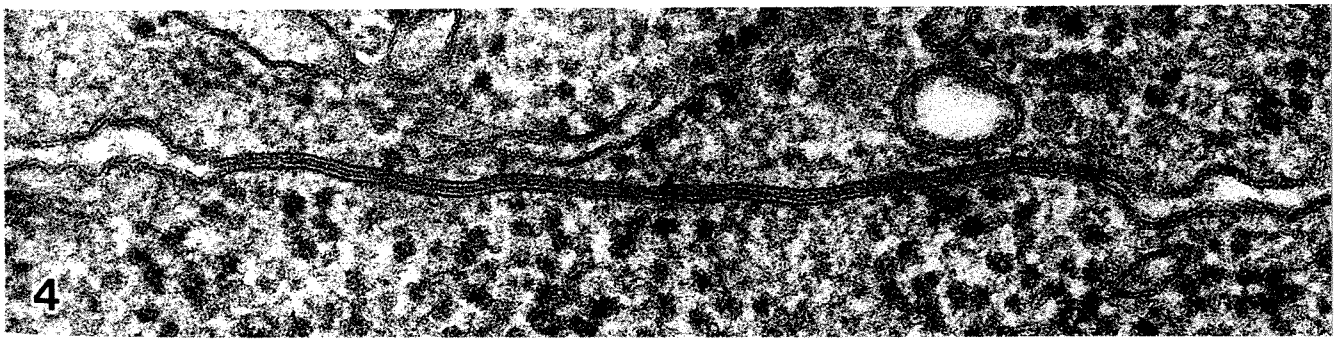
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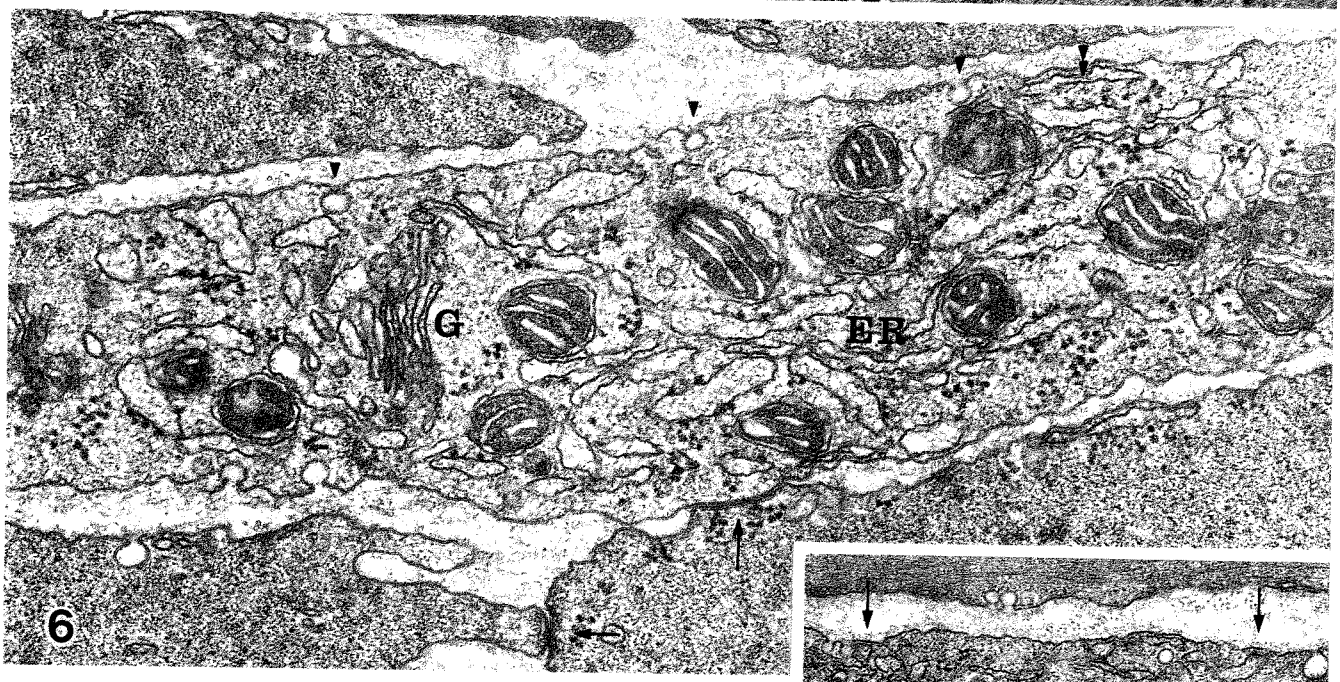
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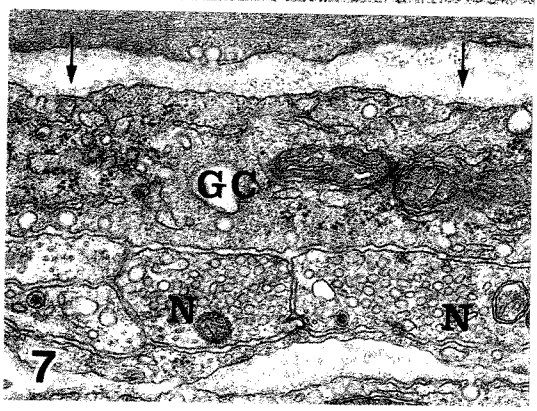
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connected to both circular and longitudinal muscle cells by gap junctions and are considered to provide morphological evidence for their electrical coupling (Komuro 1989). This type of cell probably functions in cell-to-cell communication via gap junctions in these species. Hitherto, gap junctions of fibroblast-like cells have only been reported in the tissues described above; however, further studies may disclose their existence in a wider variety of species.

An important observation is that the rat DMP is also associated with the cells characterized by many large gap junctions; these are similar to the gap-junction-rich cells of the guinea-pig small intestine (Zhou and Komuro 1992a,b). They probably correspond to the ICC-III of the mouse DMP (Rumessen and Thuneberg 1982; Rumessen et al. 1982; Thuneberg 1982, 1989) and to the cells observed in dog DMP, although the existence of myosin filaments in the latter has been postulated (Torihashi et al. 1993). This means that the cells characterized by many large gap junctions are consistently located in close association with the DMP of these animals.

On the other hand, the SMP of the colon is comparable to the DMP of the small intestine (Rumessen and Thuneberg 1982) and morphological similarities between the ICC of the dog SMP and ICC-III of the mouse DMP (Rumessen et al. 1982; Thuneberg 1982,1989) have been pointed out by Berezin et al. (1988). The latter authors have observed complexes of nerves, ICC and smooth muscle cells at the submucosal border of the dog colon and have suggested that they provide a structural basis for the pacemaker function. This suggestion is supported by the recording of slow waves from the cat colon (Du and Conklin 1989; Conklin and Du 1990). More recently, Liu et al. (1993, 1994) have demonstrated that selective damage to the ICC of the dog colon is correlated with the selective loss of slow wave activity caused by selective uptake methylene blue and subsequent intense illumination. These authors suggest that the ICC, which are characterized by numerous gap junctions interconnecting the same type of cells and neighbouring circular muscle cells, play an essential role in the generation of slow waves. Meanwhile, the significance of intercellular connection by gap junctions for the generation of slow waves has been shown by experiments using heptanol, which reversibly dissociates gap junctions (Huizinga et al. 1988; Serio et al. 1991).

These observations indicate that the cells characterized by many large gap junctions are regular components around (at or under) the submucosal interface of the circular muscle layer in the intestinal tract, and that they contribute to the generation of slow waves. The gap-junction-rich cells found in the rat DMP are also probably involved in pacemaking activity. In this context, it is interesting to consider whether human intestines have a different mechanism, since cells characterized by many large gap junctions have not previously been observed in the relevant human tissue (Rumessen et al. 1992, 1993).

Fig. 5. A cross-section of the gap-junction-rich cell (*GC*). *Arrows* indicate gap junctions with the processes of the same type of cells (*P*) and with neighbouring muscle cell (*M*). Naked axons (*N*) are in close contact with the cell. A Golgi apparatus (*G*) is located in the paranuclear region. Numerous mitochondria are also seen. ×15000

Fig. 6. An electron micrograph showing the paranuclear cytoplasm of a gap-junction-rich cell. Well-developed GER (*ER*), a Golgi apparatus (*G*) and many mitochondria are present. Caveolae (*arrowheads*) and a structure similar to the subsurface cistern (*double arrowhead*) can be seen. *Arrows* indicate gap junctions between two muscle cells and between the muscle cell and the gap-junction-rich cell. ×46000

Fig. 7. Axon terminals (*N*) containing clear rounded vesicles that are closely associated with the gap-juntion-rich cell (*GC*). *Arrows* indicate the basal lamina. ×28000

Fig. 8. A slender cell process of the gap-junction-rich cell forming large gap junctions with muscle cells of the inner (*IM*) and the outer (*OM*) sublayers (*arrows*). Caveolae and microtubules can be seen. A part of the submucosal fibroblast is found at the *upper edge* of the micrograph. ×22000

References

Anderson CR, Edwards SL (1993) Subunit b of cholera toxin labels interstitial cells of Cajal in the gut of rat and mouce. Histochemistry 100:457–464

Berezin I, Huizinga JD, Daniel EE (1988) Interstitial cells of Cajal in canine colon: a special communication network at the inner border of the circular muscle. J Comp Neurol 273:42–51

Cajal SR (1893) Sur les ganglions et plexus nerveux de l'intestin. C R Soc Biol 45:217–223

Cajal SR (1911) Histologie du système nerveux de l'homme et des vertébrés. Tome 2, Maloine, Paris

Christensen J (1992) A commentary on the morphological identification of interstitial cells of Cajal in the gut. J Auton Nerv Syst 37:75–88

Conklin JL, Du C (1990) Pathways of slow-wave propagation in proximal colon of cats. Am J Physiol 258:G894–903

Du C, Conklin JL (1989) Origin of slow waves in the isolated proximal colon of the cat. J Auton Nerv Syst 28:167–178

Huizinga JD, Shin A, Chow E (1988) Electrical coupling and pacemaker activity in colonic smooth muscle. Am J Physiol 255:C653–C660

Komuro T (1982) The interstitial cells in the colon of the rabbit. Scanning and transmission electron microscopy. Cell Tissue Res 222:41–51

Komuro T (1989) Three-dimensional observation of the fibroblast-like cells associated with the rat myenteric plexus, with special reference to the interstitial cells of Cajal. Cell Tissue Res 255:343–351

Komuro T, Zhou DS, Tokui K (1991) Cytological analysis of the interstitial cells of Cajal. J Smooth Muscle Res 27:199–202

Liu LWC, Thuneberg L, Daniel EE, Huizinga JD (1993) Selective accumulation of methylene blue by interstitial cells of Cajal in canine colon. Am J Physiol 264:G64–73

Liu LWC, Thuneberg L, Huizinga JD (1994) Selective lesoning of interstitial cells of Cajal by methylene blue and light leads to loss of slow waves. Am J Physiol 266:G485–496

Rumessen JJ, Thuneberg L (1982) Plexus muscularis profundus and associated interstitial cells. I. Light microscopical studies of mouce small intestine. Anat Rec 203:115–127

Rumessen JJ, Thuneberg L, Mikkelsen HB (1982) Plexus muscularis profundus and associated interstitial cells. II.Ultrastructure studies of mouce small intestine. Anat Rec 203:129–146

Rumessen JJ, Mikkelsen HB, Thuneberg l (1992) Ultrastructure of interstitial cells of Cajal associated with deep muscular plexus of human small intestine. Gastroenterology 102:56–68

Rumessen JJ, Peters S, Thuneberg L (1993) Light- and electron microscopical studies of interstitial cells of Cajal and muscle cells at the submucosal border of human colon. Lab Invest 68:481–495

Serio R, Barajas-Lopez C, Daniel EE, Berezin I, Huizinga JD (1991) Slow-wave activity in colon: role of network of submucosal interstitial cells of Cajal. Am J Physiol 260:G636–645

Shuttleworth CW, Xue C, Ward SM, Vente J de, Sanders KM (1993) Immunohistochemical localization of 3',5'-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. Neuroscience 56:513–522

Thuneberg L (1982) Interstitial cells of Cajal: intestinal pacemaker cells? Adv Anat Embryol Cell Biol 71:1–130

Thuneberg L (1989) Interstitial cells of Cajal. In: Wood JD (ed) Handbook of Physiology. The gastrointestinal system, vol 1. American Physiological Society, Bethesda, Maryland, pp 349–386

Torihashi S, Kobayashi S, Gerthoffer WT, Sanders KM (1993) Interstitial cells in deep muscular plexus of canine small intestine may be specialized smooth muscle cells. Am J Physiol 265:G638–645

Xue C, Ward SM, Shuttleworth CW, Sanders KM (1993) Identification of interstitial cells in canine proximal colon using NADH diaphorase histochemistry. Histochemistry 99:373–384

Xue C, Pollock J, Schmidt HHHW, Ward SM, Sanders KM (1994) Expression of nitric oxide synthase immunoreactivity by interstitial cells of the canine proximal colon. J Auton Nerv Syst 49:l–14

Young HM, McConalogue K, Furness JB, Vente J de (l993) Nitric oxide targets in the guinea-pig intestine identified by induction of cyclic GMP immunoreactivity. Neuroscience 55: 583–596

Zhou DS, Komuro T (1992a) Interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine, with special reference to the interstitial cells of Cajal. Cell Tissue Res 268:205–216

Zhou DS, Komuro T (1992b) The cellular network of interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine. Anat Embryol 186:519–527

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Anti-c-*kit* protein immunoreactive cells corresponding to the interstitial cells of Cajal in the guinea-pig small intestine

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Anti-c-*kit* protein immunoreactive cells corresponding to the interstitial cells of Cajal in the guinea-pig small intestine

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Abstract

Interstitial cells of Cajal (ICC) of the guinea-pig small intestine were studied with whole-mount preparations by using the zinc iodide–osmic acid method (ZIO) and immunohistochemistry for vimentin and c-*kit* receptor tyrosine kinase, and by electron microscopy. The myenteric ICC visualized with ZIO staining are immunopositive to both anti-c-*kit* antibody (ACK-2) and anti-vimentin antibody (V9), and constitute an independent cellular network from the myenteric plexus. Those cells are characterized by many mitochondria, abundant intermediate filaments, and surface cell membranes not covered with a basal lamina. They are connected with each other by gap junctions at tips of the cytoplasmic processes. It is concluded that the myenteric ICC of the guinea-pig intestine are fibroblast-like cells and that they correspond to the c-*kit* expressing cells regarded as the intestinal pacemaker.

Keywords: c-*kit*; Pacemaker; Ultrastructure; Interstitial cells of Cajal; Myenteric plexus; Guinea-pig

1. Introduction

During the last decade following the proposal of the pacemaker hypothesis [26], physiological studies have accumulated evidence that interstitial cells of Cajal (ICC) [1] are involved in the generation of the slow waves which represent the electrical signals of the pacemaker function in the gut peristaltic movement [4,7,8,16,17,21–23]. Therefore, these cells have become central for an understanding of intestinal movement, although the cytological definition and the developmental origin of ICC remain unsettled. While a wide variety of candidate cells have been reported in different portions of the alimentary tract in different species, including humans [5,20], the issue of the identification of ICC and pacemaker cells has been confused (see reviews [3,15] and [27]).

On the other hand, recent studies suggest that the cells expressing c-*kit* receptor tyrosine kinase in the mouse small intestine have pacemaking activity and that they correspond to ICC, by demonstrating that its genetic defect [9,28] or the experimental blockade of its function [18,25] interferes with the normal development of this type of cells, with a loss of normal propagating phasic contraction. Since these results have been derived from only limited materials or from a combination of mutant and control mice, it appeared worth investigating other animals such as the guinea-pig used in previous studies [1,24] to elucidate the intrinsic nature of ICC.

The present study intends to clarify whether these c-*kit* expressing cells truly correspond to ICC by using the zinc iodide–osmic acid (ZIO) method [19] and vimentin immunostaining [12,14] which have been used as a useful tool to identify ICC.

2. Materials and methods

2.1. Zinc iodide–osmic acid (ZIO) staining

Under terminal anaesthesia with ether, short segments of small intestine were removed from young adult (aged 4–6 weeks) guinea-pigs of both sexes and placed in a tyrode solution containing 1 mM papaverine for about 10 min at an initial temperature of 37°C [19]. Then, they were moderately inflated and fixed for 24 h at room temperature with fixative containing 0.4% OsO_4 and 2.4% ZnI_2. The

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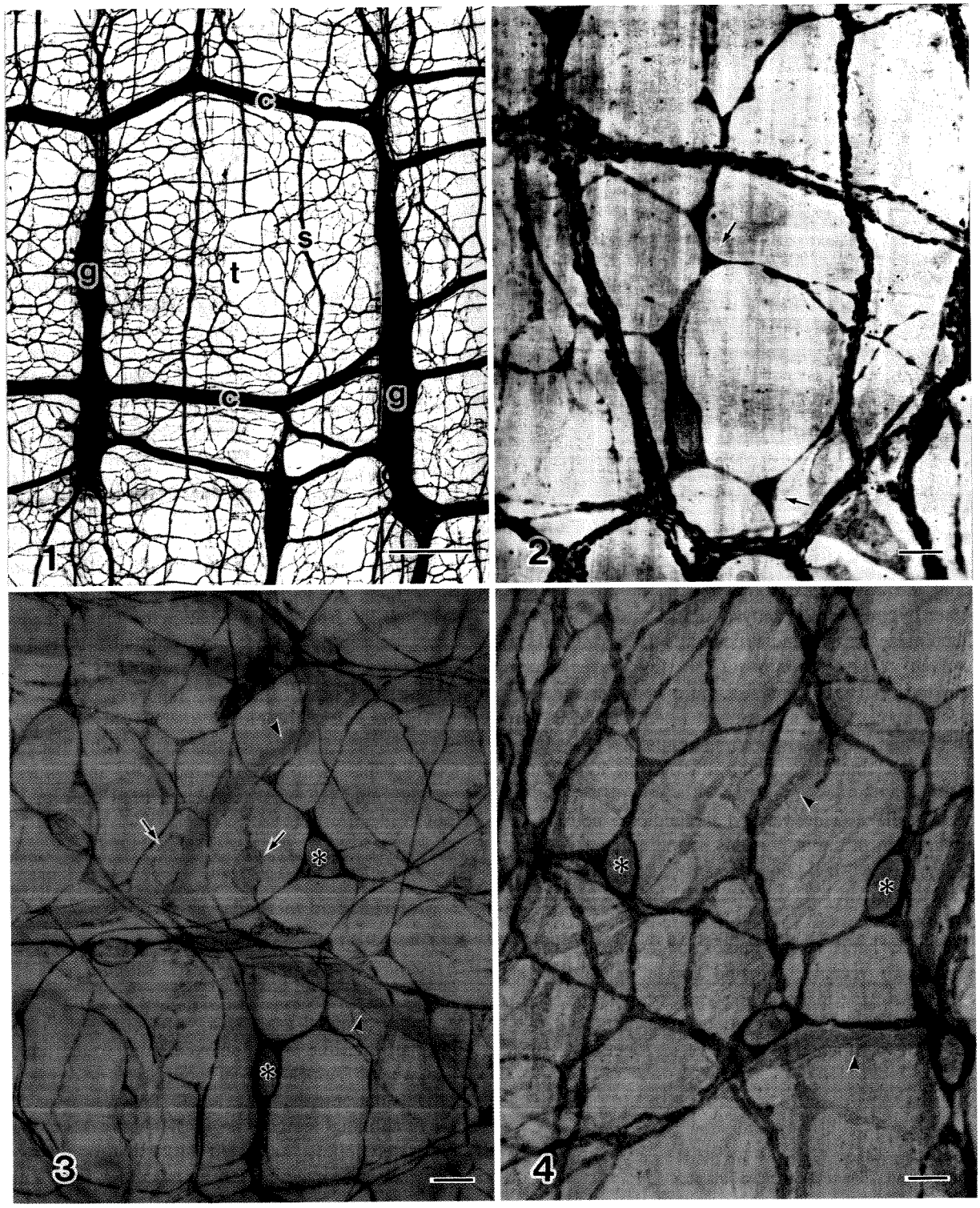
specimens were then rinsed in distilled water and cut along the mesentery to make flat sheets. Under a dissecting microscope, muscle layers with the myenteric plexus were carefully laminated with fine forceps. Those isolated pieces were distended and mounted with aqueous mounting medium for light microscopic examination.

2.2. Immunohistochemistry

Segments of guinea-pig small intestine were inflated and fixed for either 2 h at 4°C with fixative containing 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for vimentin, or 30 min at 4°C with absolute acetone for c-*kit*



receptor. After rinsing in phosphate-buffered saline (PBS), the isolated muscle layers as described above were placed in PBS containing 0.3% Triton X-100 at 4°C for 5–10 min. The specimens were then stretched on slide glass and were first incubated with 4% Block Ace solution (Dainippon Seiyaku) for 30 min at room temperature to avoid non-specific staining. The following steps for immunoreaction were separately performed for different antibodies as follows: (1) specimens were incubated with the monoclonal antibody against vimentin (DAKO,V9; No. M725) at a dilution ratio of 1:20. After washing in PBS several times, the specimens were incubated further with peroxidase-conjugated secondary antibodies (DAKO, rabbit anti-mouse IgG) at a dilution ratio of 1:100. Horseradish peroxidase reaction was developed in a solution of 6 mg of 4-chloro-1-naphthol (CN; Sigma) in 50 ml 0.1 M TRIS–HCL buffer (pH 7.4) with 8 μl of 30% H_2O_2. (2) The isolated muscle layers were incubated with the monoclonal antibody against the c-*kit* protein (ACK-2; GIBCO, No 3314SA) at a dilution ratio of 1:200. The peroxidase-conjugated secondary antibodies (goat anti-rat IgG, GIBCO 13860-010) were used at a dilution ratio of 1:80 and the peroxidase reaction was performed using the same procedure as described above. (3) Control specimens were processed in a similar manner, but the primary incubation solution did not contain anti-vimentin or ACK-2.

2.3. Cholinesterase reaction

Counter-staining of the myenteric plexus in the immunohistochemical preparations was performed using the thiocholin method [11] after the horseradish peroxidase reaction. The specimens were incubated for about 30 min at 37°C in a medium containing 5 mg acetylthiocholine iodide, 0.5 ml 0.1 M sodium citrate, 1 ml 30 mM $CuSO_4$, 1 ml distilled water and 1 ml 5 mM potassium ferricyanide in 6.5 ml 0.1 M acetate buffer (pH 6.0).

2.4. Transmission electron microscopy

Pieces of guinea-pig small intestine were placed in a fixative containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. The specimens were then rinsed in the same buffer and post-fixed in 1% OsO_4 in the same buffer for 2 h at 4°C. Following osmication, the specimens were rinsed in distilled water, block-stained with a saturated aqueous uranyl acetate solution for 2 h, dehydrated in a graded series of ethyl alcohols, and embedded in Epon Epoxy resin. Ultrathin sections were cut using a Reichert ultramicrotome and double-stained with uranyl acetate and lead tartrate for observation under a JEOL JEM 1200EX II electron microscope.

3. Results

The myenteric plexus of the guinea-pig small intestine is well depicted by the ZIO method and consists of primary, secondary and tertiary plexus (Fig. 1). ICC in the myenteric region are located among the interstices of the nerve network (Fig. 2). They are characterized by spindle or triangular cell bodies with an elongated nucleus and have three to five slender primary cytoplasmic processes. These processes usually repeat the dichotomy and often project the processes for extremely long distances. Therefore, even a single cell often spans an area of several hundreds of microns. They interconnect with each other to form the cellular reticulum, which is independent from the nerve network. One of the most peculiar features of ICC is the formation of triangular knots at every branching point of the processes.

These ICC are strongly immunopositive to anti-vimentin antibody and show almost exactly the same features (Fig. 3) as those stained with the ZIO method, which indicates that those cells are identical to the latter. Another type of cell, which shows weak immunoreactivity to vimentin, is also seen, mainly along the nerve network.

The peculiar form characterized by dichotomous slender processes and the triangular knots is also visualized by immunostaining for c-*kit* receptor (Fig. 4). Their cell bodies and the cytoplasmic processes are usually observed to be slightly thicker than those stained by anti-vimentin antibody, probably because of contours of the cell surfaces

Fig. 1. Myenteric plexus of the guinea-pig small intestine stained with the ZIO method. The fine network of the tertiary plexus (t) is observed in the interstices of the primary plexus constituted of thick ganglion strands (g) and perpendicular connecting strands (c). Several nerve bundles of the secondary plexus (s), which directly connect the primary strands, are seen. ZIO staining. Bar, 100 μm (×110).

Fig. 2. The myenteric ICC characterized by long cytoplasmic processes which show a dichotomous branching pattern. Note triangular knots at branching points (arrows). The cell body is usually seen apart from the nerve bundles. ZIO staining. Bar, 10 μm (×700).

Fig. 3. The myenteric ICC revealed by immunohistochemical staining for vimentin (*). Note the dichotomous branching pattern of the processes and the triangular knots at the branching points. Nerve bundles of the tertiary plexus are brown from the cholinesterase reaction (arrowheads). Independence of the cellular reticulum from the plexus is clear. Weakly-stained cells correspond to the fibroblasts in the myenteric region (arrows). Bar, 10 μm (×650).

Fig. 4. The myenteric ICC (*) demonstrated by immunohistochemical staining for anti-c-*kit* protein (ACK-2). The tertiary nerve plexus is brown from the cholinesterase reaction (arrowheads). Bar, 10 μm (× 650).

instead of the intermediate filaments in the cytoplasm, and partly because of different fixation effects. Their cellular reticulum is in large part independent from the nerve plexus.

Under the electron microscope, their whole cell shape, including cell bodies and long processes, can hardly be observed in a single profile, since ICC have well-demarcated cell bodies and slender processes. The perinuclear portion of ICC is often observed to be elongated and surrounded by poor cytoplasm, adapted to the narrow space between the two muscle layers (Fig. 5). However, their cytoplasm contains a well-developed Golgi apparatus, smooth endoplasmic reticulum (SER) and many mitochondria (Figs. 5 and 6). Rough endoplasmic reticulum (RER) is fairly well-developed, but its cisterns usually do not show a dilated form, unlike those of typical fibroblasts.

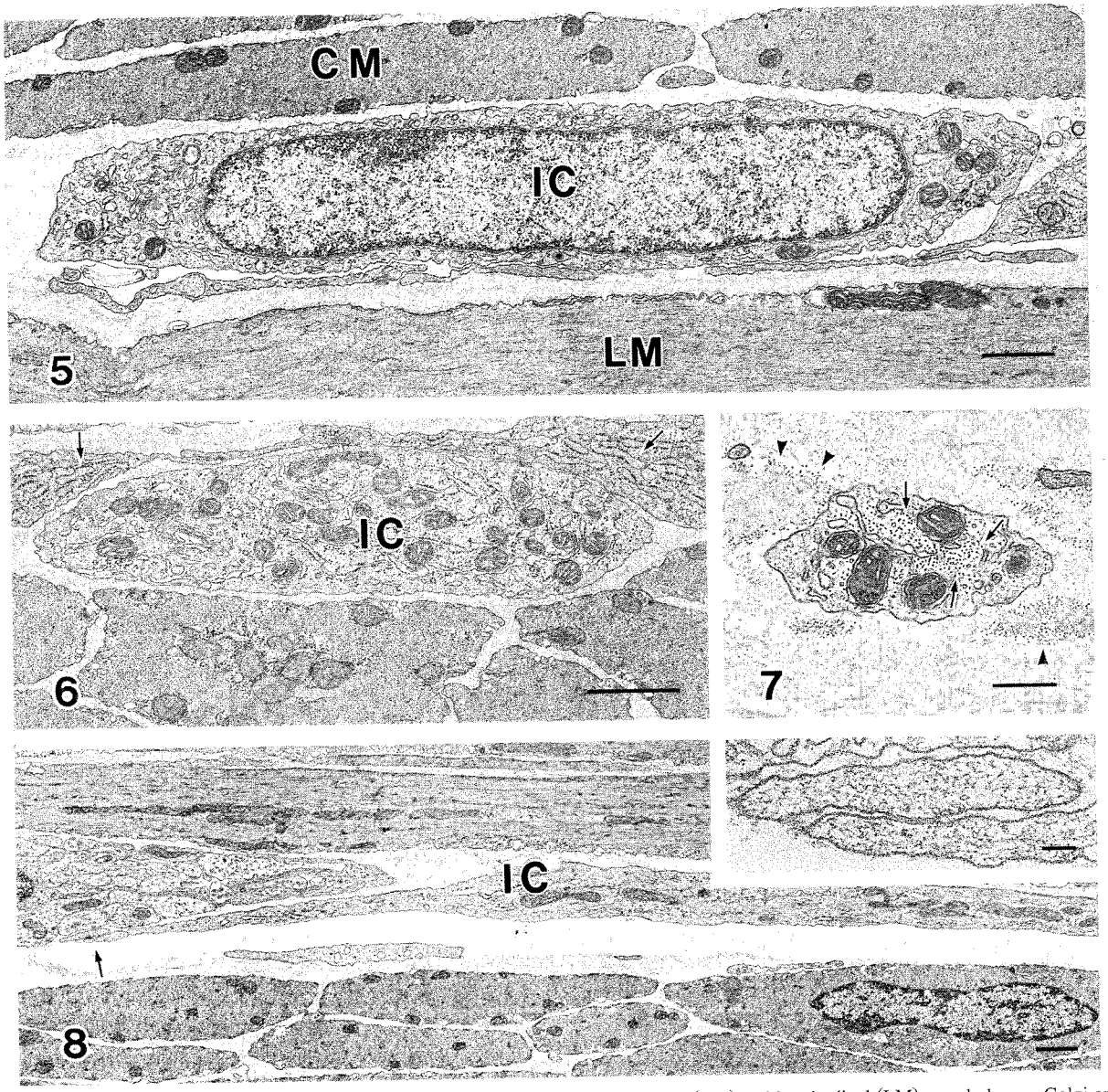


Fig. 5. An electron micrograph showing the myenteric ICC (IC) located between the circular (CM) and longitudinal (LM) muscle layers. Golgi apparatus and cisterns of SER are observed in the paranuclear cytoplasm. There is no basal lamina around the cell membrane. Bar, 1 μm ($\times$12,000).

Fig. 6. A cross section of the cytoplasmic processes of the myenteric ICC (IC) containing abundant mitochondria, SER and cisterns of RER. Dilated cisterns of RER are observed in the processes of adjacent fibroblasts (arrows). Bar, 1 μm ($\times$14,500).

Fig. 7. A thinner portion of the ICC process which contains bundles of intermediate filaments (arrows). Elastic filaments are often found in close vicinity (arrowheads). Bar, 0.5 μm ($\times$24,000).

Fig. 8. A cytoplasmic process of ICC (IC) which forms a gap junction at the tip (arrow). Bar, 1 μm ($\times$7000). Inset: Higher magnification of the gap junction indicated by the arrow. Bar, 0.1 μm ($\times$57,000).

Abundant intermediate filaments are found in the cytoplasm, particularly in the thinner portion of the processes (Figs. 7 and 8). They constitute gap junctions with each other mainly at the tips of the processes (Fig. 8 and its inset). The surface cell membrane is not covered with a basal lamina.

Another population of interstitial cells, probably representing typical fibroblasts, is found in the myenteric region. They are characterized by similar to but slightly different ultrastructures from those of ICC, and their cytoplasm shows relatively higher electron-density and contains far well-developed RER often showing dilated cisterns. Small number of macrophages and leukocytes are also seen. It is worth noting that a cell type covered with a basal lamina is never observed in the interstitium between two muscle layers of the guinea-pig small intestine.

4. Discussion

c-*kit* is a proto-oncogene encoding the receptor tyrosine kinase and is allelic with the dominant white spotting (W) locus [2,6]. Maeda et al. [18] reported that injection of a monoclonal antibody (ACK-2) against c-*kit* protein into neonatal mice caused abnormal gut movement which was accompanied by a reduction of c-*kit* expressing cells, possibly ICC, in the myenteric region. This observation was confirmed by a similar experiment showing a loss of electrical rhythmicity parallel with developmental impairment of ICC [25].

Ward et al. [28] revealed that electrical slow waves were always recorded in the intestinal muscle strips of wild-type (+/+) siblings, but were not detected in W/W^v mutant mice, which express reduced activity of tyrosine kinase. Further, Huizinga et al. [9] demonstrated that a network of c-*kit* expressing cells in a preparation of whole-mount RNA in situ hybridization, was present in the intestine of wild-type mice, but was absent in W/W^v mice. A cellular network stained with methylene blue was also absent in W/W^v mice.

The present observation clearly demonstrated the cellular reticulum consisting of characteristic features of cells by immunostaining for the c-*kit* receptor tyrosine kinase. The dichotomous branching pattern of their processes, and the formation of the cellular network closely resembles the myenteric ICC of rabbit [1] and guinea-pig [24], both stained with methylene blue. Then, almost exactly the same shape of cells as the c-*kit* immunopositive cells were also shown by the ZIO method, sharing the staining property with methylene blue that costains nerve fibers and ICC [15,24,27].

Therefore, it is very likely that the myenteric ICC of the guinea-pig small intestine correspond to c-*kit* expressing cells and function as the intestinal pacemaker cells, as in the mouse. This speculation is comparable with the results that W^s/W^s mutant rats showed abnormalities in the ileal movement and pyloric sphincter function due to a deficiency of c-*kit* expressing cells [10].

On the other hand, these peculiar features of ICC were also demonstrated by the immuno-staining for vimentin. Although antivimentin antibody (V9) potentially labels several types of cell including smooth muscle cells, endothelial cells, lymphocytes and glial cells in addition to fibroblasts, the characteristic features of the vimentin-positive cells in the present study do not conform to morphology of the former fours, in point of their cell shape, arrangement and location. Further, it must be pointed out that the electron microscopic observation demonstrated only fibroblast-like interstitial cells except a few free cells in the interstitium between two muscle layers, despite the fact that strong immunoreactivity to both anti-vimentin and anti-c-*kit* antibodies were observed in the space between two muscle layers in cryostat sections (unpublished data). Thus, it can be said that the myenteric ICC expressing c-*kit* correspond to vimentin-positive cells which coincide with the cells characterized by ultrastructures of fibroblasts.

Their strong immunoreactivity to anti-vimentin antibody on the whole-mount preparations is consistent with the observation of abundant intermediate filaments in ultrathin sections. This conclusion is also compatible with the fact that ZIO-stained cells of re-embedded preparations of the myenteric region from the guinea-pig small intestine are fibroblast-like cells [29].

Since those cells are characterized by rich cell organelles, abundant intermediate filaments and no basal lamina, the myenteric ICC of the guinea-pig small intestine seem to belong to the family of fibroblasts which has been thoroughly demonstrated to show a wide variety of morphological and functional diversities depending on their own micro-environment that are peculiar to local conditions [13].

References

[1] Cajal, S.R., Histologie du Systeme Nerveux de l'Homme et des Vertebres, Vol. 2. Paris, Maloine, 1911.
[2] Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P. and Bernstein, A., The proto-oncogene c-*kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus, Nature, 335 (1988) 88–89.
[3] Christensen, J.A., Commentary on the morphological identification of interstitial cells of Cajal in the gut, J. Auton. Nerv. Syst., 37 (1992) 75–88.
[4] Du, C. and Conklin, J.L., Origin of slow waves in the isolated proximal colon of the cat, J. Auton. Nerv. Syst., 28 (1989) 167–178.
[5] Faussone-Pellegrini, M.S., Pantalone, D. and Cortesini, C., Smooth muscle cells, interstitial cells of Cajal and myenteric plexus interrelationships in the human colon, Acta Anat., 139 (1990) 31–44.
[6] Geissler, E.N., Ryan, M.A. and Housman, D.E., The dominant white spotting (*W*) locus of the mouse encodes the c-*kit* protooncogene, Cell, 55 (1988) 185–192.
[7] Hara, Y., Kubota, M. and Szurszewski, J.H., Electrophysiology of smooth muscle of the small intestine of some mammals, J. Physiol., 372 (1986) 501–520.

[8] Huizinga, J.D. Shin, A. and Chow, E., Electrical coupling and pacemaker activity in colonic smooth muscle, Am. J. Physiol., 255 (Cell Physiol., 24) (1988) C653–C660.
[9] Huizinga, J.D., Thuneberg, L., Kluppel, M., Malysz, J., Mikkelsen, H.B. and Bernstein, A., *W / kit* gene required for interstitial cells of Cajal and for intestinal pacemaker activity, Nature, 373(26) (1995) 341–349.
[10] Isozaki, K., Hirota, S., Nakama, A., Miyagawa, J., Shinomura, Y., Xu, Z.D., Nomura, S. and Kitamura, Y., Disturbed intestinal movement, bile reflux to the stomach, and deficiency of c-*kit*-expressing cells in *Ws / Ws* mutant rats, Gastroenterology, 109(2) (1995) 456–463.
[11] Karnovsky, M.J. and Roots, L., A direct-coloring thiocholine method for cholinesterase, J. Histochem. Cytochem., 12 (1964) 219.
[12] Komuro, T., Three-dimensional structure of the intestinal wall, Ther. Res., 7 (1987) 107–115.
[13] Komuro, T., Re-evaluation of fibroblasts and fibroblast-like cells, Anat. Embryol., 182 (1990) 103–112.
[14] Komuro, T., Zhou, D.S. and Tokui, K., Identification of the interstitial cells of Cajal (Abstracts). XIV Federative Int. Congr. of Anat. (1994) p. 169.
[15] Komuro, T., Tokui, K. and Zhou, D.S., Identification of the interstitial cells of Cajal, Histol. Histopathol. 11 (1996) 769–786.
[16] Lee, H.K. and Sanders, K.M., Comparison of ionic currents from interstitial cells and smooth muscle cells of canine colon, J. Physiol. (London), 460 (1993) 135–152.
[17] Liu, L.W.C., Thuneberg, L. and Huizinga, J.D., Selective lesioning of interstitial cells of Cajal by methylene blue and light leads to loss of slow waves, Am. J. Physiol., 266 (Gastrointest. Liver Physiol., 29) (1994) G485–G496.
[18] Maeda, H., Yamagata, A., Nishikawa, S., Yoshinaga, K., Kobayashi, S., Nishi, K. and Nishikawa, S., Requirement of c-*kit* for development of intestinal pacemaker system, Development, 116 (1992) 369–375.
[19] Rumessen, J.J. and Thuneberg, L., Plexus muscularis profundus and associated interstitial cells. I. Light microscopical studies of mouse small intestine, Anat. Rec., 203 (1982) 115–127.
[20] Rumessen, J.J. and Thuneberg, L., Interstitial cells of Cajal in human small intestine. Ultrastructural identification and organization between the main smooth muscle layers, Gastroenterology, 100 (1991) 1417–1431.
[21] Serio, R., Huizinga, J.D., Barajas-Lopez, C. and Daniel, E.E., Interstitial cells of Cajal and slow wave generation in canine colonic circular muscle, J. Auton. Nerv. Syst., 30 (1990) S141–S144.
[22] Smith, T.K., Spontaneous junction potentials and slow waves in the circular muscle of isolated segments of guinea-pig ileum, J. Auton. Nerv. Syst., 27 (1989) 147–154.
[23] Suzuki, N., Prosser, C.L. and Dahms, V., Boundary cells between longitudinal and circular layers: Essential for electrical slow waves in cat intestine, Am. J. Physiol., 250 (Gastrointest. Liver Physiol., 13) (1986) G287–294.
[24] Taxi, J., Contribution a l'etude des connexions des neurones moteurs du systeme nerveux autonome, Ann. Sci. Nat. Zool., VII (1965) 413–474.
[25] Torihashi, S., Ward, S.M., Nishikawa, S., Nish, K., Kobayashi, S. and Sanders, K.M., c-*kit* dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract, Cell Tissue Res., 280 (1995) 97–111.
[26] Thuneberg, L., Interstitial cells of Cajal: Intestinal pacemaker cells? Adv. Anat. Embryol. Cell Biol., 71 (1982) 1–130.
[27] Thuneberg, L., Interstitial cells of Cajal. In J.D. Wood (Ed.), Handbook of Physiology. The Gastrointestinal System, Vol 1. American Physiology Society, Bethesda Maryland, 1989, pp. 349–386.
[28] Ward, S.M., Burns, A.J., Torihashi, S. and Sanders, K.M., Mutation of the proto-oncogene c-*kit* blocks development of interstitial cells and electrical rhythmicity in murine intestine, J. Physiol. (London), 480 (1994) 91–97.
[29] Zhou, D.S. and Komuro, T., Ultrastructure of the zinc iodide–osmic acid stained cells in guinea pig small intestine, J. Anat., 187 (1995) 481–485.

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Histology and Histopathology

Invited Review

Identification of the interstitial cells of Cajal

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Summary. Observation of whole-mount stretch preparations using the zinc-iodide-osmic acid method reveals a wide variety of interstitial cells in different tissue layers of the guinea-pig small intestine. And a subsequent electron microscopic examination and survey of references makes clear that the interstitial cells of Cajal (ICC) depicted in original drawings of Cajal are heterogeneous and correspond to different types of interstitial cells. The myenteric ICC are characterized by long dichotomous branching processes which constitute cellular networks independent from the nerve plexus and form many gap junctions at their tips. Their ultrastructure is similar to that of fibroblasts and they have no basal lamina. The myenteric ICC show strong immunoreactivity for vimentin and the *c-kit* receptor, and probably correspond to the intestinal pacemaker cells.

Within the circular muscle layer, ICC are represented by the cells that are closely associated with fine nerve bundles. The ICC have various shapes, ranging from bipolar to stellate, depending on the running pattern of the nerve fibers that they are associated with. They show fibroblast-like ultrastructure and have no basal lamina. They form gap junctions with smooth muscle cells and are immunoreactive for vimentin.

On the other hand, ICC associated with the deep muscular plexus described in the guinea-pig by Cajal could not be clearly identified. However, it is suggested that the ICC in this location may correspond to glycogen-rich cells possessing a basal lamina. Although they show a fairly well-developed rough endoplasmic reticulum, Golgi apparatus and immunoreactivity for vimentin, ICC of the deep muscular plexus are probably specialized smooth muscle cells in nature.

Key words: Ultrastructure, *c-kit*, Vimentin, Intestine, Pacemaker

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Introduction

The great neuroanatomist, Santiago Ramon y Cajal described «cellules interstitielles (or neurones sympathiques interstitiels)» in association with the terminal arborization of the autonomic nerves of intestines, glands and blood vessels stained with methylene blue or the Golgi method (Cajal, 1893, 1911). Ever since then, interstitial cells of Cajal (ICC), as referred to by following microscopists, have been a subject of a historical debate with respect to their cytological nature. The cells located in the interstitium of the intestinal wall, in particular, have received special attention by many investigators (see reviews by Boeke, 1949; Meyling, 1953; Taxi, 1965).

Among others, Taxi (1965) maintained the opinion that ICC were neural in nature and were distinct from fibroblasts, whereas Richardson (1958, 1960) was sceptical about the presence of two distinct cell types and suggested that ICC were fibroblasts, based on observations of silver impregnation of the rabbit small intestine.

Modern ICC research was revived by Thuneberg (1982) who proposed the novel hypothesis that ICC act as pacemakers and have an impulse conductive function in the intestinal musculature analogous to those of the heart muscle, as suggested earlier by Keith (1914/15, 1915, 1916). Indeed, recent physiological studies have accumulated evidence that ICC are involved in the generation of the slow waves which represent the electrical signals of the pacemaker function (Hara et al., 1986; Suzuki et al., 1986; Huizinga et al., 1988; Du and Conklin, 1989; Conklin and Du, 1990; Serio et al., 1990; Liu et al., 1993, 1994). Consequently, these cells have become central for an understanding of intestinal movement.

However, the cytological definition and the developmental origin of ICC remain unsettled. Part of the confusion seems to result from a variety of cells observed in different tissue layers in different portions of the alimentary tract, in different species (see reviews by Thuneberg, 1989; Christensen, 1992). Another important reason for the uncertainty is the lack of a truly specific

staining method for ICC. In view of these problems, Thuneberg (1982, 1989) advanced the understanding of ICC by making an extensive survey of the literature, and by the classification of these cells on the basis of their location and ultrastructure. Christensen (1992) raised several significant questions about ICC, attempting to answer them by making a broad survey of the literature, but was able to establish only rather vague cytological criteria for ICC.

More recently, several new approaches to identifying ICC by (immuno)histochemical methods including NADH diaphorase histochemistry (Xue et al., 1993), Chorela toxin subunit b labelling (Anderson and Edwards, 1993), cyclic GMP immunoreactivity (Shuttleworth et al., 1993; Young et al., 1993) and NO synthase immunoreactivity (Xue et al., 1994) have been developed. However, again, none of them have been considered as being truly specific for ICC.

When ICC research enters a new phase, such as to discuss a certain gene (*c-kit*) in association with intestinal pacemaker activity (Ward et al., 1994; Huizinga et al., 1995), it is essential to establish a set of clear-cut criteria for ICC to support the further development of ICC research. For instance, studies of cytodifferentiation of ICC (Faussone-Pellegrini, 1984, 1985, 1987) must be understood for establishing the morphological criteria of matured specimens.

To exclude ambiguity from a definition of ICC, it is important to identify them on the basis of a good correlation between a given cell type and those originally described by Cajal. Therefore, first of all, the present study elucidates the whole cell shape of a variety of interstitial cells located in different tissue layers in the guinea-pig small intestine by using whole-mount stretch preparations stained by a modified zinc iodide-osmic acid (ZIO) method (Rumessen and Thuneberg, 1982).

The ZIO method is not a specific staining for ICC: rather it stains nerve fibers and a certain range of interstitial cells which are likely to include cells with the features of ICC. This property of the ZIO method is shared with methylene blue and the Golgi method, which were originally used to observe ICC (Cajal, 1893, 1911). It is therefore a useful tool for identifying ICC. Good penetration ability is another advantage of the ZIO method, which makes it possible to observe any tissue layer of the whole-mount preparations of guinea-pig small intestine.

Immunohistochemistry for vimentin and the c-*kit* can also be applied to speculate about the developmental origin and functional role of ICC. Vimentin immunostaining has been used as a useful tool to observe ICC (Komuro, 1987; Komuro et al., 1994).

Further, we clarified the ultrastructural features of candidate cells by means of a critical correlation of their whole cell shapes with the original drawings of ICC (Cajal, 1893, 1911). This approach makes it possible to define clear-cut morphological criteria for ICC via integrating observations obtained by traditional histological staining and by modern methods.

Regarding nomenclature, the term ICC will be used only for those cells whose equivalence to the original description has been confirmed, or those which can be regarded as species variations of ICC. The rest of the cells, including those which have been regarded as ICC in the literature, without firm evidence, and the cells of the interstitium in a general sense, will be described simply as «interstitial cells» to avoid confusion.

In the following text, ICC at different locations will be described according to the terminology of Thuneberg et al. (1995) i.e., ICC-AP (Auerbach plexus) located between the circular and longitudinal muscle layers; ICC-DMP (deep muscular plexus) located between the inner thin and outer thick sublayers of the circular smooth muscle of the small intestine; ICC-SMP (submuscular plexus) located at the submucosal border of the colonic circular muscle layer; ICC-CM located within the outer thick circular muscle layer; and ICC-LM located within the longitudinal muscle layer. Cells in the subserous tissue layer are described simply as interstitial cells for the reason described above.

This article addresses only cytological identification of ICC and readers are advised to consult comprehensive review articles by Thuneberg (1982, 1989), Christensen (1992) and Thuneberg et al. (1995) for other aspects of ICC. A part of this study has been published elsewhere (Komuro et al., 1994).

Materials and methods

Zinc iodide-osmic acid (ZIO) staining

Short segments of guinea-pig small intestine were placed in a Tyrode solution containing 1mM papaverine to make a complete relaxation of the muscle cells for about 10 min, at an initial temperature of 37 °C (Rumessen and Thuneberg, 1982). Then, they were moderately inflated and fixed for 24 h at room temperature with fixative containing 0.4% OsO_4 and 2.4% ZnI_2. The specimens were then rinsed in distilled water and cut along the mesentery to make flat sheets. Under a dissecting microscope, circular muscle layers, longitudinal muscle layers, with or without the myenteric plexus, and serosa without serous epithelium were carefully dissected from remaining parts of the intestinal wall with fine forceps. These specimens were mounted with aqueous mounting medium Mount Quick (Daido Sangyo). For transmission electron microscopy, suitable areas of the specimens were cut out, block-stained with a saturated aqueous uranyl solution for 2 h and then processed for electron microscopy as described below.

Immunohistochemistry

Short segments of guinea-pig small intestine were inflated and fixed for either 2 h at 4 °C with fixative containing 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for vimentin and S-100, or 30 min at

4 °C with 100% acetone for c-*kit* receptor. After rinsing in phosphate-buffered saline (PBS) each layer of specimen was dissected as described above. The isolated pieces were placed in PBS containing 0.3% Triton X-100 at 4 °C for 5-10 min. The specimens were then stretched on a glass slide and were first incubated with 4% Block Ace solution (Dainippon Seiyaku) for 30 min at room temperature to avoid non-specific staining. The following steps for immunoreaction were separately performed for different antisera as follows: **vimentin-** Specimens were incubated overnight with the monoclonal antibody against vimentin (DAKO, V9; No. M725) at a dilution of 1:20. After washing in PBS several times, the specimens were incubated further overnight with peroxidase-conjugated secondary antibodies (DAKO, rabbit anti-mouse IgG) at a dilution of 1:100. Horseradish peroxidase reaction was developed in a solution of 6mg of 4-chloro-1-naphtol (CN; Sigma) in 50 ml 0.1M Tris-HCl buffer (pH 7.4) with 8 µl of 30% H_2O_2. **c-kit receptor-** The isolated muscle layers were incubated overnight with the monoclonal antibody against the c-kit receptor (ACK-2; GIBCO, No 3314SA) at a dilution of 1:200. The peroxidase-conjugated secondary antibodies (goat anti-rat IgG, GIBCO 13860-010) were used at a dilution of 1:80 and the peroxidase reaction was performed with the same procedure as described above. **S-100-** Specimens were incubated overnight with anti-S-100 antibody (DAKO, Z311, rabbit polyclonal) at a dilution of 1:200. The primary antibody was visualized with biotin-streptavidin method by using BAS-PO kit (Biogenex Lab, San Ramon).

Cholinesterase reaction

Counterstaining of the myenteric plexus in the immunohistochemical preparations was performed by thiocholine method (Karnovsky and Roots, 1964) after the horseradish peroxidase reaction. In brief, the specimens were incubated for about 30 min at 37 °C in medium containing 5mg acetylthiocholine iodide, 0.5 ml 0.1M sodium citrate, 1ml 30mM $CuSO_4$, 1 ml distilled water and 1ml 5mM potassium ferricyanide in 6.5 ml 0.1M acetate buffer, pH 6.0.

Bauer's staining for glycogen

Short segments of guinea-pig small intestine were moderately inflated and fixed for 4 h with Bouin's fixative. After rinsing in PBS, the circular muscle layer was dissected, as described above. The specimens were stained by Bauer's glycogen staining (using Chromic acid instead of Periodic acid in the PAS reaction) to avoid coloration of the basal lamina of the smooth muscle cells around the target interstitial cells.

Transmission electron microscopy

Pieces of guinea-pig small intestine were placed in a fixative containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for 2 h at 4 °C. The specimens were then rinsed in the same buffer and post-fixed in 1% OsO_4 in the same buffer for 2 h at 4 °C. Following osmication, the specimens were rinsed in distilled water, block-stained with a saturated aqueous uranyl acetate solution for 2 h, dehydrated in a graded series of ethyl alcohols, and embedded in Epon Epoxy resin. Ultrathin sections were cut using a Reichert ultramicrotome and double-stained with uranyl acetate and lead tartrate for observation with a JEOL JEM 1200EX II electron microscope.

Results

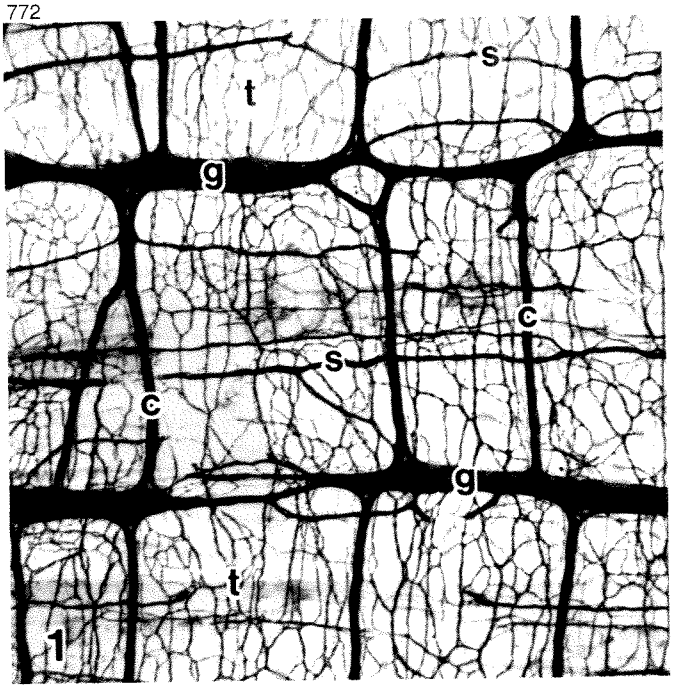
Cell shapes revealed by whole-mount stretch preparations

Region of the myenteric plexus

The myenteric plexus of the guinea-pig small intestine consists of three components: the primary, secondary and tertiary plexus (Fig. 1). Interstitial cells were generally stained using longer incubation times with ZIO and appeared in the interstices of the tertiary meshwork (Fig. 2). They were easily distinguished from Schwann cells which were located in the midst of nerve bundles (Fig. 3). Four types of cells were recognized using the ZIO method.

Cells of the first type were well stained using the ZIO method and usually showed darker gray cytoplasm with paler nuclei (Fig. 4). They were irregularly shaped cells with fairly large perinuclear cytoplasm and a few, broad primary processes with sharp, wavy contours. The nuclei were elongated with a longer diameter of about 12-18 µm and a shorter diameter of about 6-10 µm. In the cytoplasm, vacuoles and fat droplets were frequently seen in counter contrast. They had a close association with nerve bundles of the tertiary plexus. Therefore, the processes did not have a regular branching pattern, and were usually confined with adjacent meshes of the tertiary plexus. However, they occasionally sent out the processes perpendicular to the plane of the myenteric plexus and penetrated into the circular muscle layer, giving off further branches running parallel to the muscle cells. Although the cells made contact with processes of the same type of cells, they did not seem to constitute their own complete cellular network. Cells of this type were weakly stained with vimentin immunohistochemistry (Fig. 8).

Cells of the second type were triangular or stellate cells with long slender processes with smooth and rather straight contours (Fig. 5). They were prominent and constituted the main population of interstitial cells in this region. They were often stained so weakly by ZIO that the cytoplasmic particulate could be seen through light gray cytoplasm. The nuclei were generally elongated and slightly larger than those of the first type, measuring about 15-20 µm by 8-10 µm. The cell bodies were



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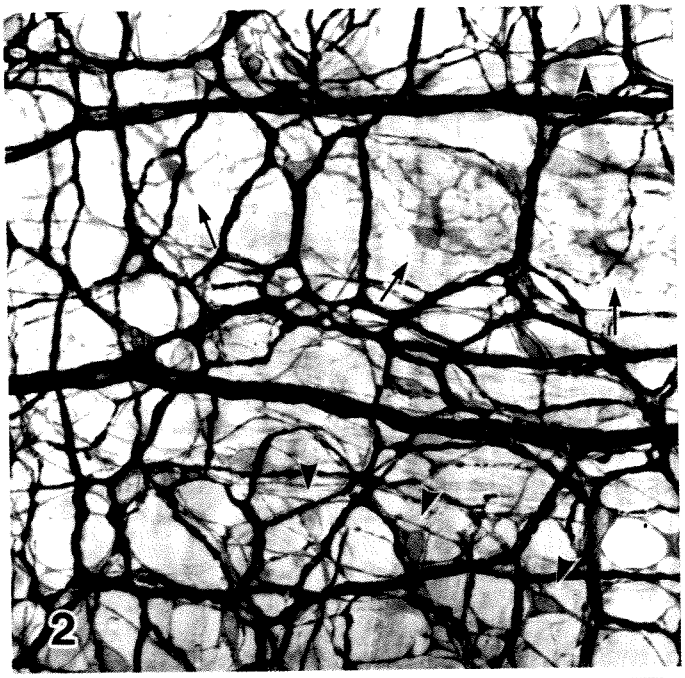
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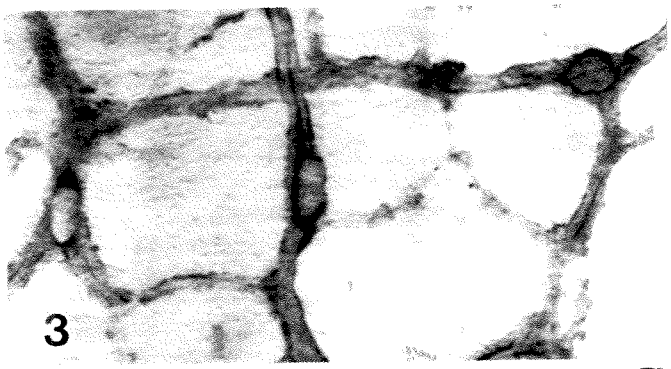
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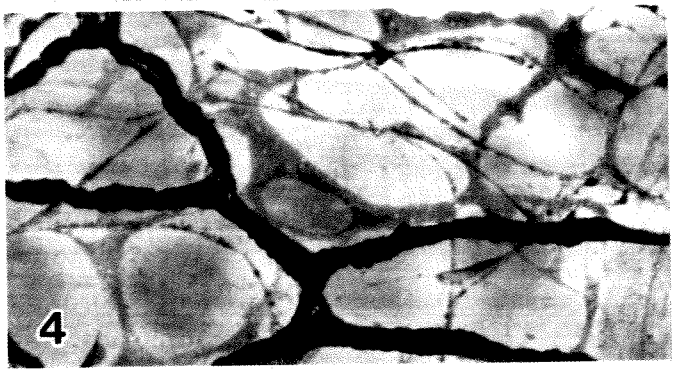
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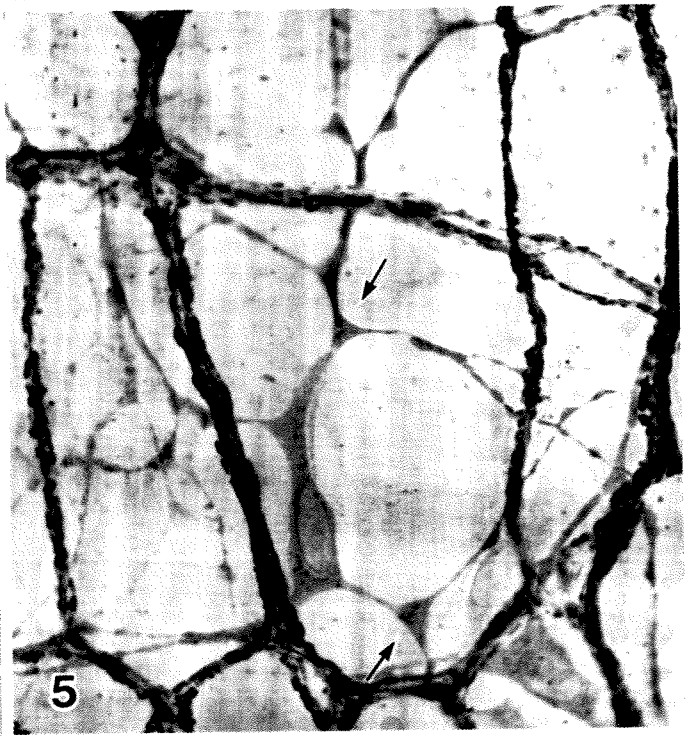
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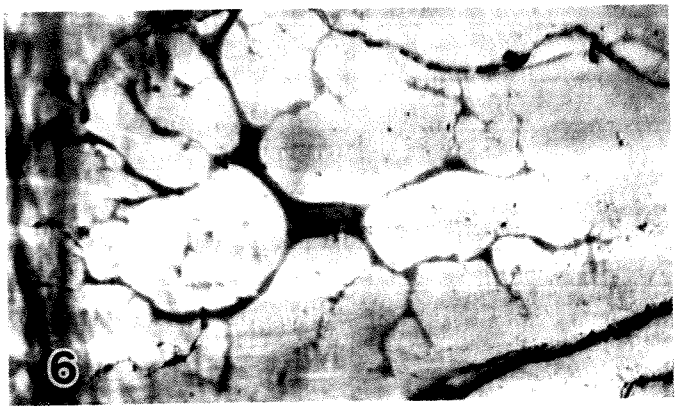
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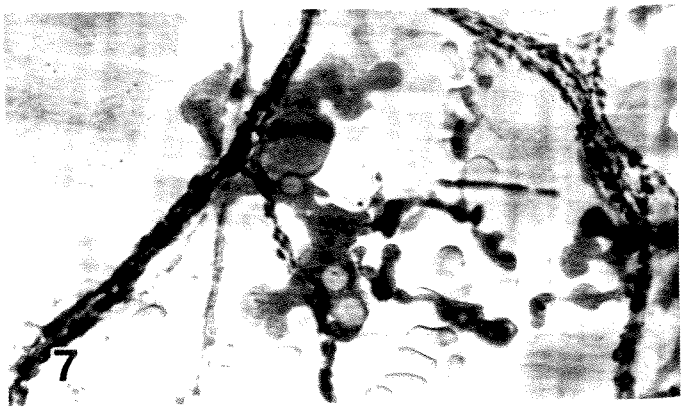
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located in meshes of the tertiary plexus and did not show close relations with nerve bundles. Three to five primary processes repeated the dichotomy to form secondary, tertiary and further branches. Therefore a single cell of this second type often spanned an area of several hundreds of microns in diameter. These processes connected with the same type of cells to form a distinct cellular network from the nerve plexus. However, in part, the processes appeared to make contacts with nerve bundles of the tertiary plexus. One of the most characteristic features of this cell type was the formation of a triangular knot at every branching point of the processes (Fig. 5). These cells were intensely stained with vimentin immunohistochemistry (Fig. 8). Cells

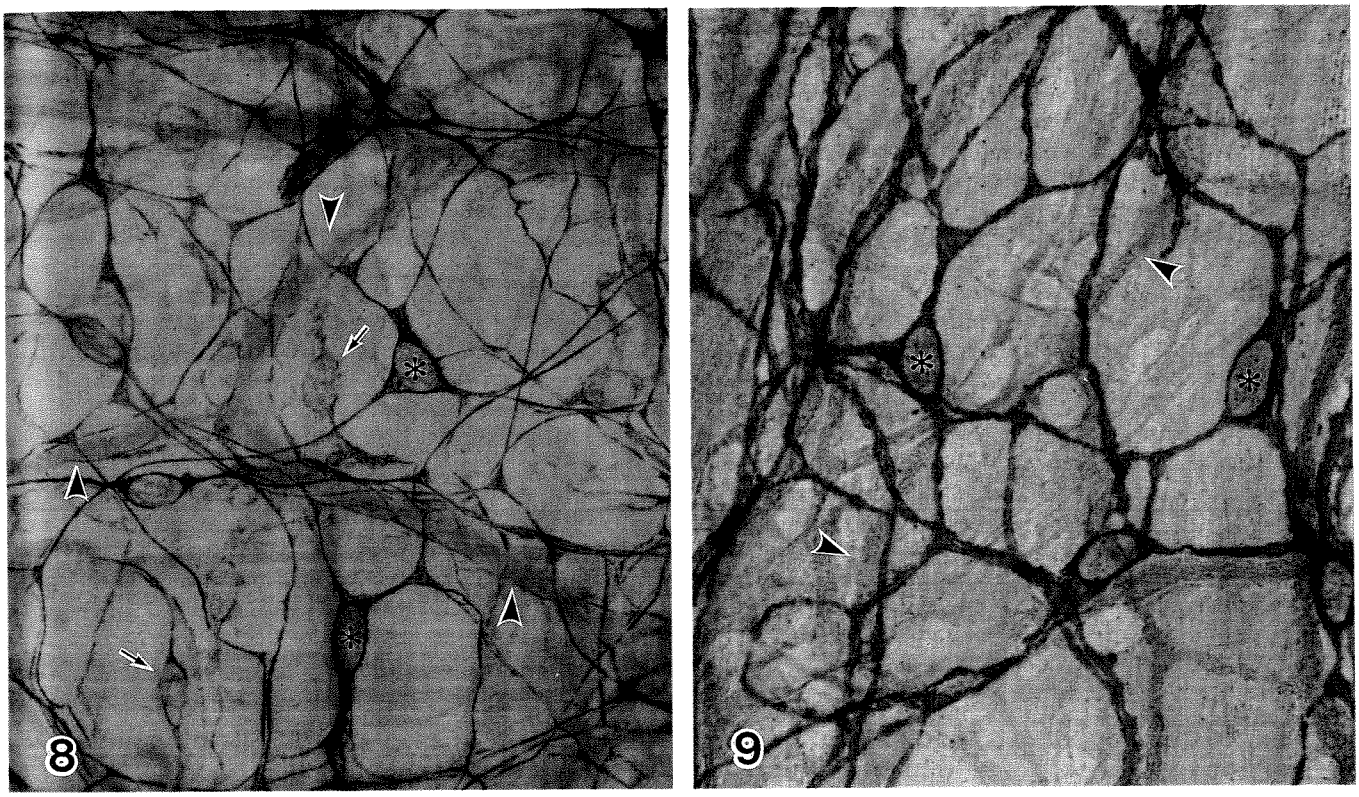


Fig. 8. The second type of myenteric interstitial cells revealed by immunohistochemical staining fro vimentin (*). Note the dichotomous branching pattern of the processes and the triangular knots at the branching points. Nerve bundles of the tertiary plexus are stained brown by the cholinesterase reaction (arrowheads). Independence of the cellular reticulum from the plexus is clear. Weakly-stained cells correspond to the first type of myenteric interstitial cells (arrows). From Komuro and Zhou, 1996. x 650

Fig. 9. Almost exactly the same shape of cells (*) as those in Fig. 8, demonstrated by immunohistochemical staining for c-kit receptor. The tertiary nerve plexus is stained brown by the cholinesterase reaction (arrowheads). From Komuro and Zhou, 1996. x 650

Fig. 1. Myenteric plexus of the guinea-pig small intestine-stained with ZIO method. The fine network of the tertiary plexus (t) is observed in the interstices of the primary plexus constituted by thick ganglion strands (g) and perpendicular connecting strands (c). Several nerve bundles of the secondary plexus (s), which directly connect the primary strands, are seen. ZIO staining. x 120

Fig. 2. A light micrograph showing interstitial cells (arrowheads) over the tertiary plexus and the cells of lymph vessels (arrows). ZIO staining. x 300

Fig. 3. Schwann cells located in the midst of the nerve bundles of the tertiary plexus stained with S-100 immunohistochemistry. x 700

Fig. 4. The first type of myenteric interstitial cells in the guinea-pig small intestine which is located beside the tertiary nerve. It extends several irregular processes. ZIO staining. x 850

Fig. 5. The second type of myenteric interstitial cell characterized by long cytoplasmic processes which show a dichotomous branching pattern. Note triangular knots at branching points (arrows). The cell body is usually seen apart from the nerve bundles. ZIO staining. x 700

Fig. 6. Stellate cells of the lymph vessel. ZIO staining. x 600

Fig. 7. A probable macrophage displaying a different appearance of inclusions revealed by ZIO staining. x 750

having a shape almost exactly similar to those of vimentin-positive cells were demonstrated by *c-kit* receptor immunostaining (Fig. 9). They also showed a dichotomous branching pattern with a triangular knots, and their cellular network was independent from the myenteric plexus.

Many lymph vessels were distributed in the myenteric region, and clusters of stellate cells were observed on the wall of lymph vessels, as confirmed with Nomarski optics (Figs. 2, 6). They were always superimposed on the lymph vessels and confined to the width of the vessels. Cells of this type were similar in size to the first type of interstitial cells, but they differed from the latter in their correlation to nerve bundles. The cell bodies contained round to elongated nuclei and projected the processes in all directions as if they enveloped the wall of the lymph vessel. The processes occasionally showed a dichotomous branching pattern, but they were easily distinguished from the second type because of their irregular contour and angular course. They made contact with each other but they did not appear to form a complete cellular network. Immunoreactivity for vimentin was not observed in cells of this type.

Cells of another type, probably representing macrophages, were scattered in the interstices of the myenteric plexus (Fig. 7). They were elongated cells with a few, short processes and were characterized by many granular structures of different appearance and size. Some of them were vacuolar and others were very dense. These cells tended to be located in the vicinity of blood vessels. They did not have vimentin immunoreactivity.

Circular muscle layer

The circular muscle coat of the guinea-pig small intestine is subdivided into inner thin and outer thick sublayers by the intercalation of the deep muscular plexus (DMP).

Within the outer circular muscle layer, nerve bundles mainly ran parallel to the muscle cells and formed a fairly well-organized plexus with few interconnecting strands (the superficial plexus: SP). Interstitial cells found within this layer were of only one type, which was always well-stained using the ZIO method (Figs 10-13). They were characterized by spindle-shaped cell bodies with a few primary processes. The nuclei were elongated and usually measured about 15 μm by 7 μm.

Their cytoplasmic processes showed different patterns of ramification depending on their relation to nerve bundles. These patterns were roughly classified into three variations, as follows: Cells of the first pattern were observed in association with long, straight portions of nerve bundles which were widely separated from neighboring bundles. They were bipolar cells with extremely long processes and kept a close relationship with nerves throughout the whole extension (Fig. 10). The processes generally showed sharp, wavy contours and a gradual decrease in the caliber towards the tips, though the terminal portions occasionally formed a complex arborization. Bipolar primary processes rarely gave off large secondary processes, but often made fine lateral twigs which penetrated into the muscle tissue.

Cells of the second pattern were seen in association with two parallel nerve bundles and formed an H shape (Fig. 11). The cell bodies attached to one bundle projected a broad primary process perpendicularly towards the neighboring bundles and then gave off bi-directional secondary processes which accompanied the latter. The secondary processes showed similar features to the primary processes of the first pattern.

Cells of the third pattern were found at or near the

Fig. 10. A fusiform interstitial cell beside a thin nerve bundle of SP within the circular muscle layer. Bipolar slender processes are closely associated with varicose nerve fibers. ZIO staining. x 620

Fig. 11. An H-shaped interstitial cell associated with two parallel nerve bundles of SP. An arrow indicates a connecting portion between the parallel processes (arrowheads). ZIO staining. x 650

Fig. 12. A stellate interstitial cell located at the intersection of SP. ZIO staining. x 620

Fig. 13. A stellate cell detached from the nerve bundles. Note that terminal portions of all processes are nearly parallel with each other, which reflects the axis of the circular muscle fibers. ZIO staining. x 620

Fig. 14. A stellate cell within the circular muscle layer demonstrated by the immunohistochemistry for vimentin. x 700

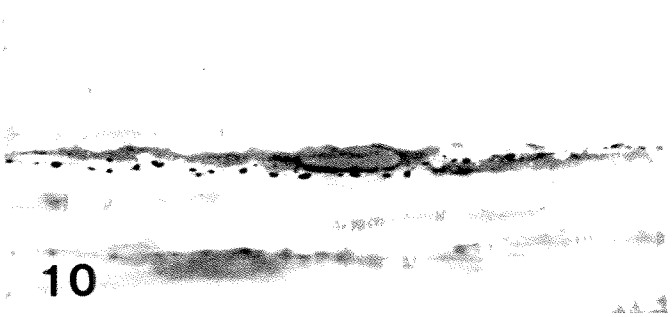
Fig. 15. A drawing of ICC within the rabbit circular muscle stained with methylene blue, adopted from Cajal (1911, Fig. 573). Note the close similarity to Figs. 13 and 14.

Fig. 16. The first type of interstitial cell in the DMP region. The cell body is located beside the nerves and extends several cytoplasmic processes along the nerve network (arrows). ZIO staining. x 850

Fig. 17. The second type of interstitial cell in the DMP region. It extends cytoplasmic processes independently from the nerve bundles (arrows). ZIO staining. x 850

Fig. 18. Immunopositive cells for vimentin antiserum in the DMP region. Note their rounded nuclei and branching processes. x 900

Fig. 19. A glycogen-rich cell stained by Bauer's staining. Arrows indicate its processes. x 800



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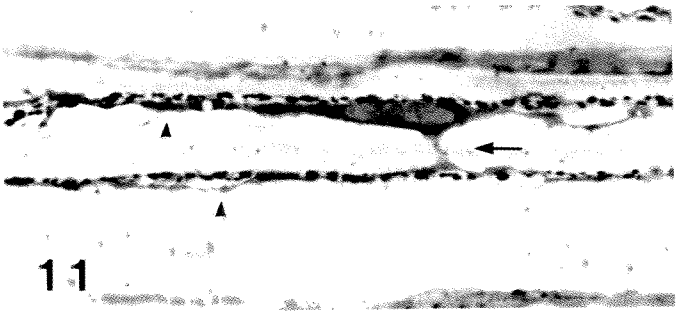

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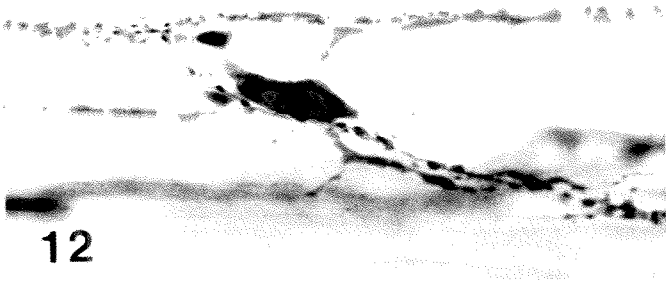

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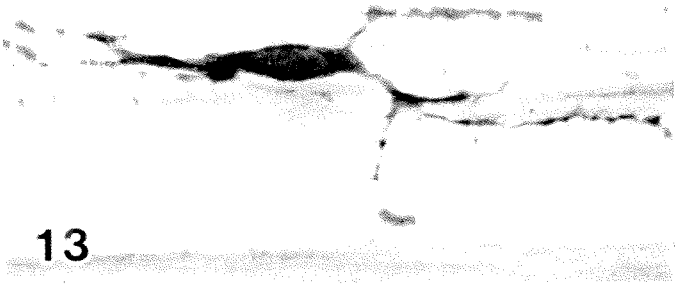

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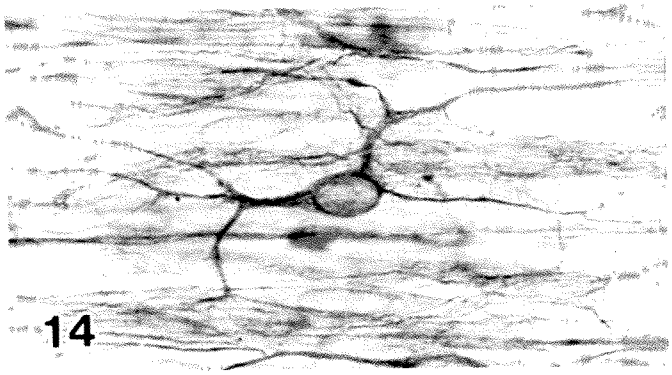

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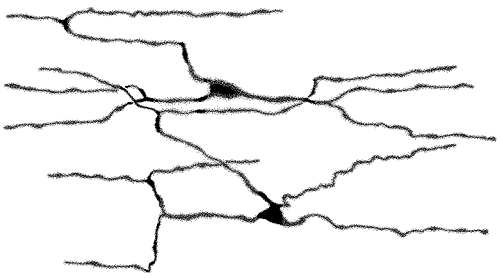

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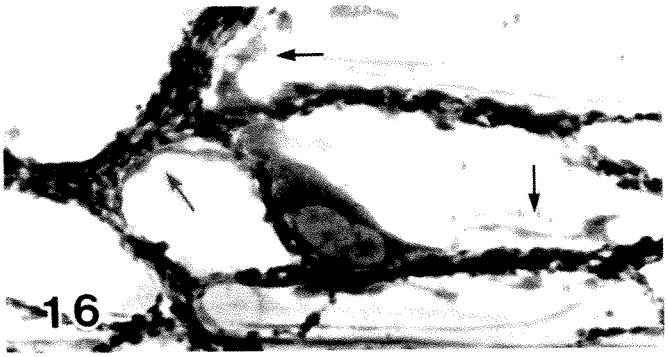

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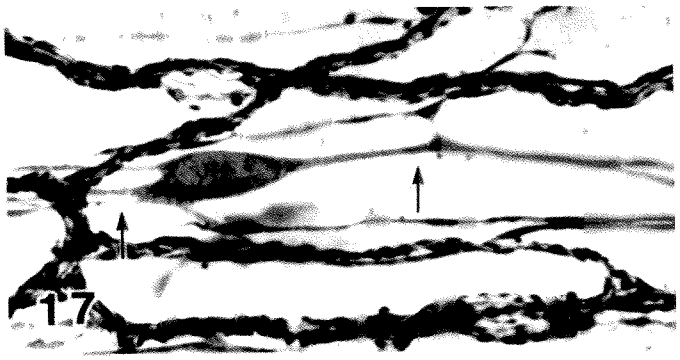

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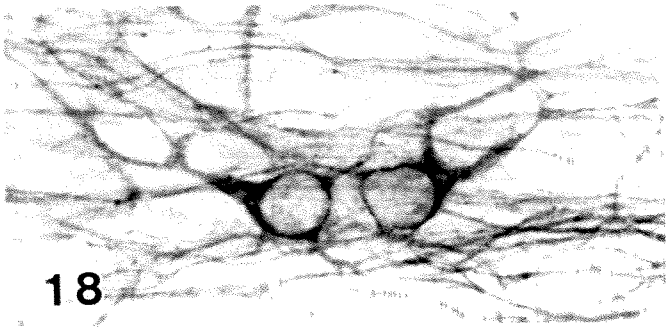

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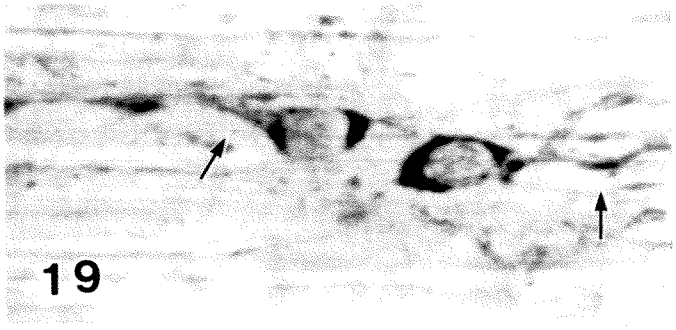

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intersections of the nerve plexus (Figs. 12, 13). They projected three to five primary processes depending on the number of nerve bundles at these sites. The processes often bridged the gap between the nerve bundles to associate with adjacent ones. Their secondary or tertiary processes occasionally extended along single varicose fibers which originated from these intersections. The whole shape of these cells was clearly observed in specimens in which a part of the nerve plexus had been detached from the cells (Fig. 13). Further, their appearance was almost exactly identical to that of the drawings by Cajal (1911) (Fig. 15). All of these variations of cells were stained with vimentin immunohistochemistry (Fig. 14).

Region of the deep muscular plexus (DMP)

The DMP is located between the inner, thin and the outer, thick sublayers of the circular muscle coat and encircles the intestinal wall. It consists of nerve bundles parallel to the circular muscle fibers and has few interconnecting bundles.

Cells of the first type in this plexus were most well stained with the ZIO method and showed darker gray cytoplasm with paler nuclei (Fig. 16). They were densely distributed throughout the whole network of the nerve bundles. The cell bodies with rich perinuclear cytoplasm were usually elongated and situated beside the nerves. They often appeared to encircle the nerve bundles. The nuclei were also elongated measuring 12-18 μm by 6-10 μm. These cells took a variety of forms depending on the sites of the associated nerve bundles. At straight portions of the nerves, they showed slim spindle shapes with long bipolar processes, while at the intersections the cells projected three to five processes along the courses of the nerves. Their broad processes showed sharp wavy contours and gradually decreased their caliber. In general, the secondary and tertiary processes were not well developed. A few fat droplets could be seen in the cytoplasm.

Cells of the second type were only occasionally observed with the ZIO method. They were spindle-shaped or polygonal cells with three to five primary processes (Fig. 17). The slender processes had smooth contours, and bifurcated and extended for extremely long distances. These processes did not have a close relationship with the nerves.

Cells unstained with ZIO method, which may represent another type of cell, were recognized from their peculiar locations and the shape of the nuclei. Nomarski optics demonstrated a regular distribution of unstained nuclei situated in small loops of nerve bundles, or at/near the intersections. They were distinguished from Schwann cells, which almost always lodge within the midst of the nerve bundles, by their larger size and by the rounded shape of the nuclei.

On the other hand, vimentin immunostaining demonstrated many positive cells with long branching processes (Fig. 18). They often occurred in pairs. They appeared independently from the nerve plexus, though their correlative positions were not clearly identified because of the difficulty of visualizing simultaneously both vimentin-positive cells and the nerve plexus. Their whole cell shape seemed to differ from either the first type or the second type of cells in the DMP. Bauer's staining, however, revealed a regular distribution of glycogen-containing cells which extended long processes (Fig. 19), resembling these vimentin-positive cells.

Along large nerve bundles interconnecting between the myenteric plexus and the superficial plexus or DMP, there were cells resembling the first type of DMP region with respect to staining affinity and close relationship to the nerves. Their features were particularly well observed in the occasional half-stained preparations of the nerves in which they enveloped the nerve bundles with their laminar cytoplasmic process (Fig. 20). A similar appearance of cells was also found in association with blood vessels in the muscle layer (Fig. 21). Here, elongated cells beside the nerve bundles extended a few slender processes along or around the blood vessels.

Longitudinal muscle layer

A small number of interstitial cells were observed in the longitudinal muscle layer with the ZIO method (Fig. 22), though there were few nerve fibers in this layer. Their cell bodies orientated nearly parallel to that of the muscle cells. The nuclei were quite elongated and often measured about 20 μm by 4 μm. These cells were characterized by small perinuclear cytoplasm with several primary processes which occasionally branched off extremely long processes. The slender processes tended to originate directly from the well-demarcated cell bodies and to extend in different directions. The processes showed sharp wavy contours and often formed secondary and tertiary extensions. Fine lateral, twigs were seen throughout the processes. Their immunoreactivity to vimentin has not been confirmed so far.

Serosa

There were many small stellate cells which were densely and regularly distributed in a two-dimensional plane in the serosa (Fig. 23). They extended many cytoplasmic processes in all directions, which branched off repeatedly. The processes did not extended for a long distance but appeared to make contact with each other. They did not show a definite cell axis, but the longer axis of their cell bodies tended to orientate parallel to that of the longitudinal muscle cells. These cells displayed strong immunoreactivity to vimentin antiserum (Fig. 24).

Discussion

The present observations revealed a wide variety of

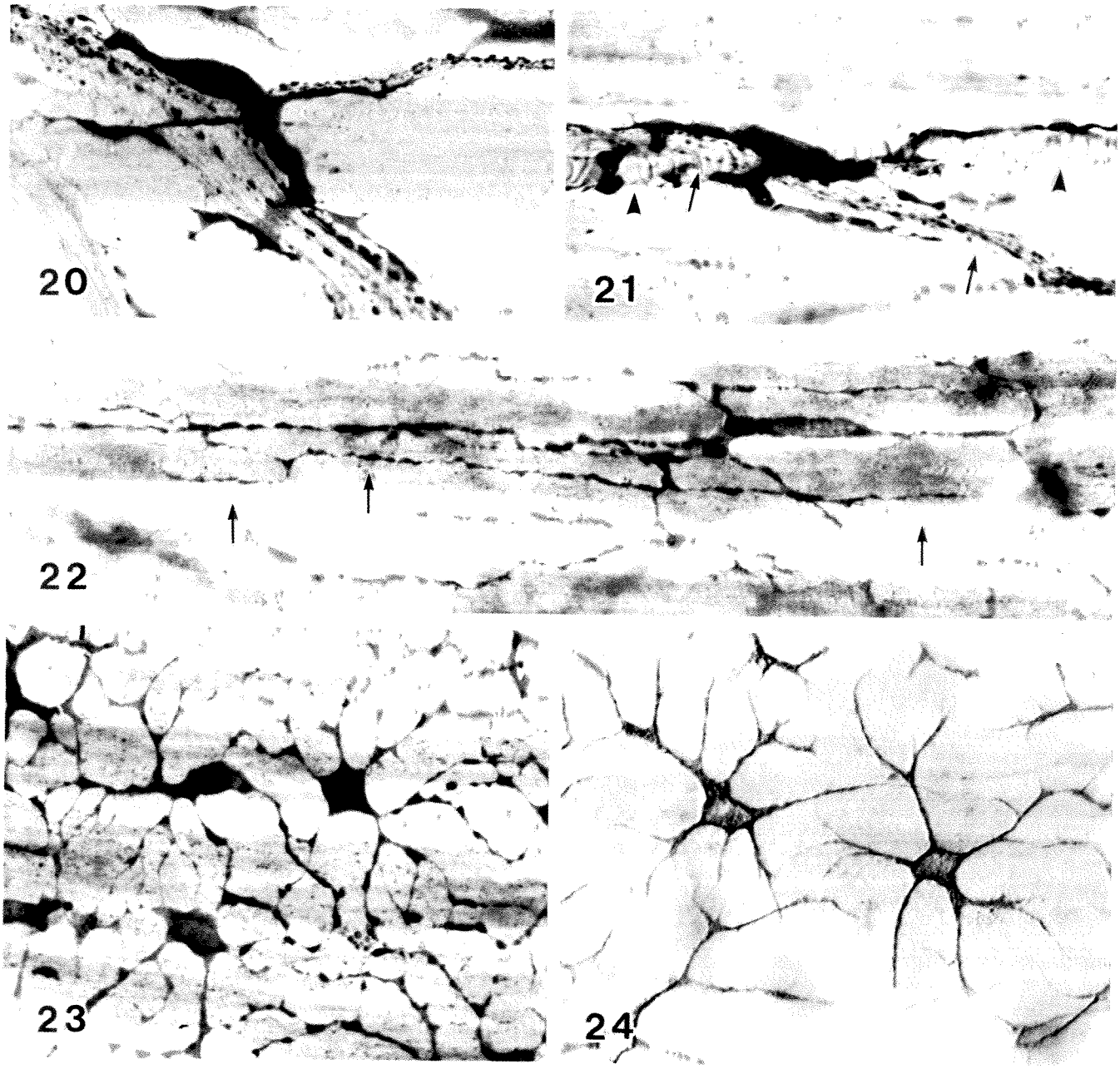


Fig. 20. An interstitial cell associated with an interconnecting nerve bundle between the myenteric plexus and SP. The cell processes envelop a weakly-stained nerve bundle. ZIO staining. x 700

Fig. 21. An interstitial cell associated with a nerve bundle (arrow) and a capillary (arrowhead) in the circular muscle layer. ZIO staining. x 700

Fig. 22. An interstitial cell in the longitudinal muscle layer, which is characterized by poor perinuclear cytoplasm and extremely long branching processes (arrow). Axis of the longitudinal muscle is horizontal. ZIO staining. x 450

Fig. 23. Interstitial cells in the serosa. They extend many branching processes in all directions. ZIO staining. x 600

Fig. 24. Interstitial cells in the serosa demonstrated by immunohistochemistry for vimentin. Their shape and organization is the same as those of Fig. 23. x 650

cells which seem to include the majority of cell types described as ICC in previous reports, by using the ZIO method which stains nerves and a range of interstitial cells. This staining property is common to that of methylene blue or the Golgi method and gives an important clue as to the identity of ICC. The correct location in a particular tissue layer of stained cells and their topographical relationship with the nerves can be identified owing to the distinctive pattern of each nerve plexus. These observations help to classify the interstitial cells on the basis of their morphology and their location.

Their existence as a particular type of cells

Before discussing ICC in detail, the fundamental question about the existence of ICC should be clarified, since it was postulated that the structures which Cajal called interstitial cells in the intestine did not originate from one cell type, but were a chimera composed of the glial cell bodies and the neurites stained simultaneously (Kobayashi et al., 1989).

The present observations clearly demonstrate the presence of several groups of cells which are distinctive in shape and size. For example, the second type in the myenteric region and the stellate cells within the circular muscle layer show their own particular morphology, which is almost identical to the original drawings by Cajal (1911, Fig. 572) and (1911, Fig. 573), respectively. Simultaneous clear visualization of cells of the same shape by vimentin immunohistochemistry (present observation; Komuro and Zhou, 1996) confirms the existence of the specific type of cells, rather than the glia-neurite chimera, because neurites do not contain a substantial amount of vimentin. Therefore, the ICC illustrated by Cajal do not represent merely artificial images, but rather a certain type of cell.

Kobayashi et al. (1986) also postulated that ICC are included in the term enteroglial cells, together with enteric glial cells and Schwann cells, on the basis of S-100 immunostaining of the guinea-pig small intestine. However, it is difficult to categorize these cells into one, since the localization of GFAP, which distinguishes their glial nature (Jessen and Mirsky, 1980), is confined within the myenteric and submucous plexuses and has not been detected on ICC and Schwann cells (Jessen and Mirsky, 1980, 1985; Bjorklund et al., 1984; Nada and Kawana, 1988).

ICC-AP

As mentioned above, the second type of cell in this region demonstrates a characteristic cell shape and forms a cellular network, similar to the ICC described by Cajal (1911, Fig. 572). It is obvious that these cells have been repeatedly observed by many light microscopists. They correspond to the cells of the guinea-pig myenteric plexus stained with methylene blue (Taxi, 1965, Fig. 48), and to the cells of the rabbit stained with silver impregnation (Richardson, 1958, Figs. 1, 3).

Electron microscopic observations revealed that only fibroblast-like cells are the proper interstitial cells (except for the free cells) in this region of the guinea-pig small intestine and that they are well stained with the ZIO method (Zhou and Komuro, 1995; Fig. 25). Their cautious examination suggests that in this region the fibroblast-like cells without a basal lamina can be classified into two subtypes (Komuro and Zhou, 1996). Cells of one type show cytoplasmic features of the typical fibroblast in many respects, but they form small gap junctions with smooth muscle cells (Figs. 26, 27).

On the contrary, cells of another type (Fig. 28) are usually identified by less electron-dense cytoplasm containing many smooth endoplasmic reticulum (sER) components and numerous mitochondria. Fairly well-developed rough endoplasmic reticulum (rER) is also found in the cytoplasm, but its cisterns rarely display dilated forms, unlike those of the former type. The electron-lucent cytoplasmic processes, which are frequently observed between two muscle layers and around the myenteric ganglia (Fig. 29), probably belong

Fig. 25. An electron micrograph showing a fibroblast-like cell (F) stained with the ZIO method between the circular (C) and longitudinal (L) muscle layers in the guinea-pig small intestine. x 9,000

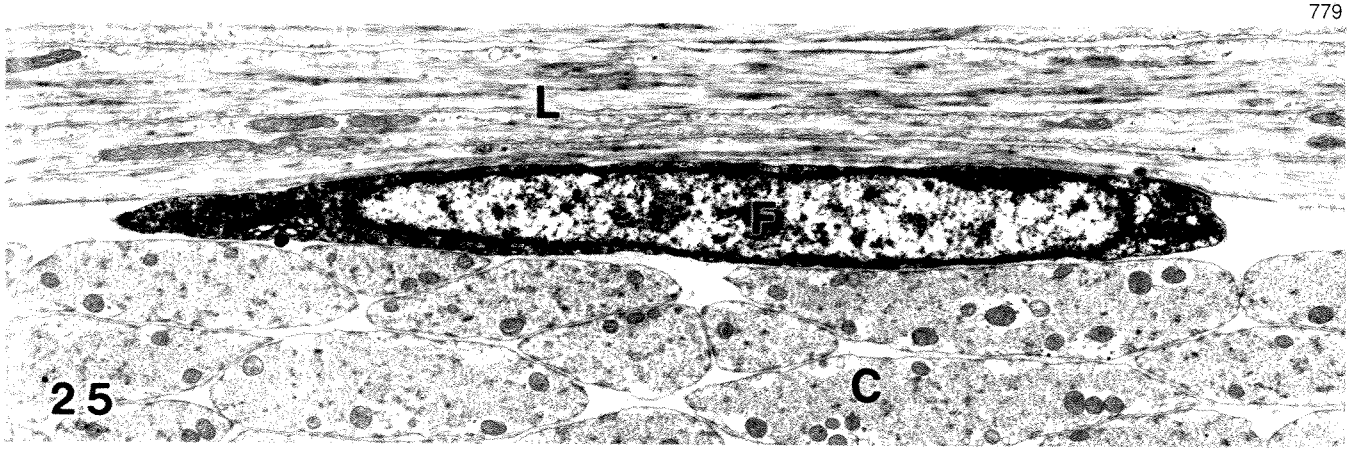
Fig. 26. One type of fibroblast-like cell (F1) in the myenteric region. it makes a small gap junction with circular muscle cells with its small process around the cell body (arrow). Cisterns of rER and several mitochondria are observed in the cytoplasm. x 7,800

Fig. 27. Higher magnification of the gap junction indicated by the arrow in Fig. 26. x 52,000

Fig. 28. Another type of fibroblast-like cell (F2) in the myenteric region of the guinea-pig small intestine, which corresponds to ICC-AP. Tubular and vesicular components of sER are observed in the perinuclear cytoplasm. rER is observed in a neighboring cell, which is probably the same type as that in Fig. 26. x 9,800

Fig. 29. A gap junction between two processes which are speculated to belong to the same type of cells as those in Fig. 28. They are found in the myenteric region and contain abundant intermediate filaments. They have no basal lamina. x 40,000

Fig. 30. An electron micrograph showing two subtypes of fibroblast-like cells (F1, F2) between the circular and longitudinal muscle layers in the rat small intestine. «F1» cells are characterized by a more electron-dense cytoplasm and well-developed rER, while «F2» cells have less electron dense cytoplasm and contain more mitochondria and sER components. A small gap junction is observed between «F1» and a tiny process of the muscle cell (arrow). x 10,000. **Insertion:** Higher magnification of the gap junction indicated by the arrow, in a neighboring section to that in Fig. 30 (a, x 31,000), and a large gap junction between two processes speculated to belong to the cell type «F2» (b, x 33,000). Modified from Komuro, 1989.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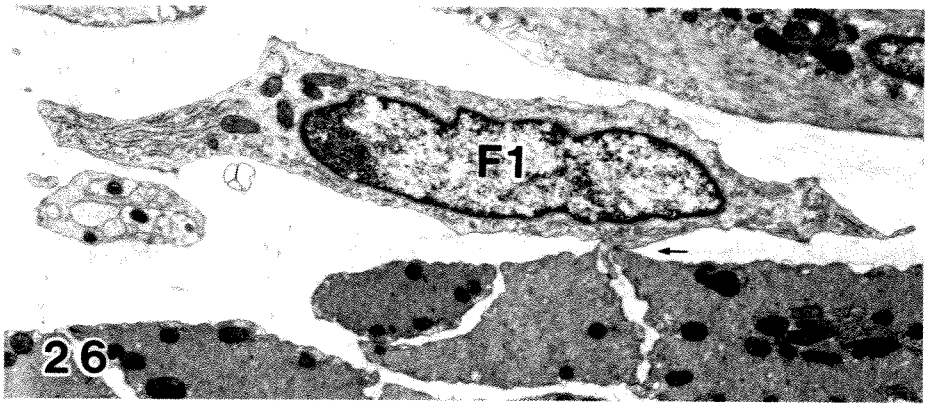
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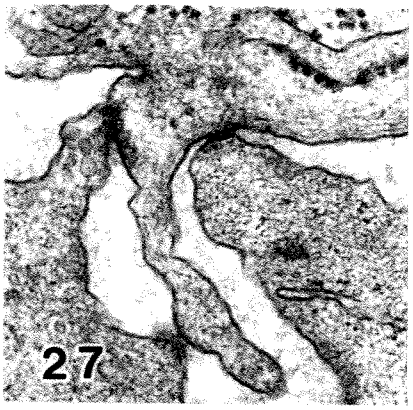
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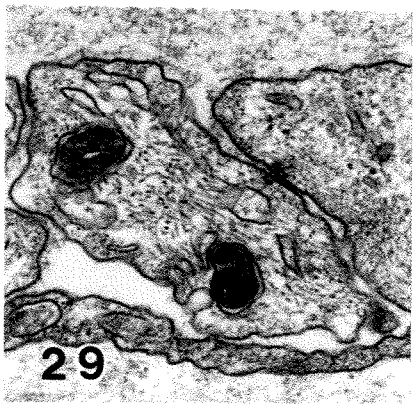
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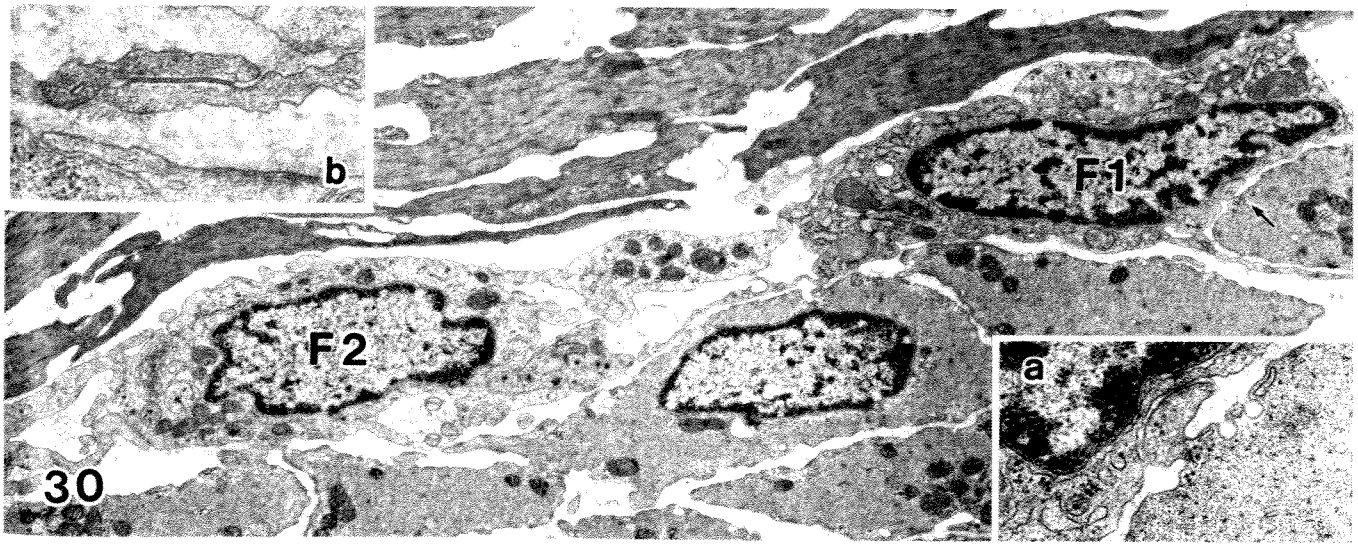
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to this type of cell, though their direct continuity has not been confirmed so far. These cytoplasmic processes are rich in intermediate filaments, and are connected with one another by fairly large gap junctions.

We speculate that the latter fibroblast-like cells correspond to the ICC in the myenteric region (ICC-AP), described by Cajal (1911), since ICC depicted by the whole mount preparations in the present study show extremely long processes and are connected with one another at their tips. These facts seem to account for the difficulty of observing the continuity between their cell bodies and the slender processes in ultrathin sections. The presence of abundant intermediate filaments in the processes also suggests that they belong to a part of the cells which show strong immunoreactivity to vimentin antiserum. Another cell subtype may correspond to the first type of cell observed by the ZIO method. Although it cannot be ruled out that they represent merely different profiles or functional states of the same type of cell, demonstration of only second type of cell by *c-kit* immunohistochemistry appears to support our speculation.

Cells showing similar ultrastructural features to each of these two subtypes were also distinguished in the myenteric region of the rat small intestine (Komuro, 1989, Fig. 30). The scanning electron micrographs showing well-demarcated cell bodies with slender processes (Komuro, 1989, Fig. 3) probably represent ICC-AP in the rat.

On the other hand, ICC-AP of the mouse intestine (Thuneberg, 1982, 1989) appear to have different fine structural features from those of the guinea-pig. They were described as having an incomplete basal lamina and showing parallel overlapping of the primary processes with nerve bundles of the plexus. Their cytoplasm seems to contain abundant intermediate filaments and numerous mitochondria.

ICC-AP of the human small intestine were reported to show myoid features, including dense bodies, caveolae, basal lamina and well-developed sER, but their gap junctions with one another and with smooth muscle were not observed (Rumessen and Thuneberg, 1991; Rumessen et al., 1993a,b). Berezin et al. (1990) described that ICC-AP of the canine colon had dark condensed cytoplasm and occasional dense bodies. Cells with myoid features were also reported in the human stomach (Faussone-Pellegrini et al., 1989).

The interstitial cells in the guinea-pig small intestine observed by SEM (Baluk and Gabella, 1987; Jessen and Thuneberg, 1991) show intimate association with nerve fascicles of the tertiary plexus, and therefore they probably correspond to the first type of cell in the present observation.

Interstitial cells of the cat intestine depicted by ZIO (Vajda and Feher, 1980) are unique in shape and arrangement and probably correspond to the cell type found on the lymph vessels in the present study. They are most likely to be adventitial fibroblasts.

ICC-CM

Examination of cell shapes found in the outer circular muscle layer offers an important clue for considering cell types and identifying ICC. The cytoplasmic processes of the stellate cells show almost the same branching pattern as those of ICC in the rabbit circular muscle layer (ICC-CM) stained by methylene blue (Cajal, 1911, Fig. 573). It is very likely that these ZIO stained cells correspond to the ICC-CM described by Cajal.

The bipolar cells observed in the present study are similar in shape to the cells of DMP of the mouse small intestine (Rumessen and Thuneberg, 1982) and the cells of the circular muscle layer of the opossum esophagus (Christensen et al., 1987), which are stained by the ZIO method. Differences between ICC-DMP and ICC-CM were stressed in the mouse small intestine (Rumessen and Thuneberg, 1982), in which bipolar cells were regarded as being peculiar to DMP. However, the bipolar cells were frequently observed in close association with varicose nerve fibers in the circular muscle layer of the guinea-pig small intestine.

Bipolar, H-shaped and stellate cells of the circular muscle layer in the guinea-pig intestine are likely to represent structural variations of the same type of cells because of similar staining with the ZIO method, similar range of nuclear size and similar topographical relationship to the nerves. Their different cell shapes appear to be determined by their locations.

Since they are stained with vimentin antiserum, and since only one cell type characterized by fibroblastic ultrastructure is found within the circular muscle layer (Zhou and Komuro, 1992b; Fig. 31), they can be regarded as fibroblast-like cells in nature. Some of these cells are quite similar in shape to the cells of the circular muscle layer of the canine colon, which are visualized by the NADH diaphorase method (Xue et al., 1993). These NADH diaphorase-positive cells were reported to lack a basal lamina.

On the other hand, two types of myoid interstitial cells were distinguished in the human small intestine in different locations within the circular muscle layer (Rumessen et al., 1993a).

ICC-DMP

The first type of cells in the DMP region were always observed beside nerve bundles, and their processes showed a close association with the running course of the nerve bundles. In this respect, they were similar to both ICC-CM, and those located along the connecting nerves between the myenteric and superficial or deep muscular plexus, in the present observation. Cells of similar features were also described in the methylene blue staining preparation by Taxi (1965, Fig. 50). However, these cells do not match the ICC-DMP of the original drawings (Cajal, 1911) in all significant points such as the location of the cell bodies and the

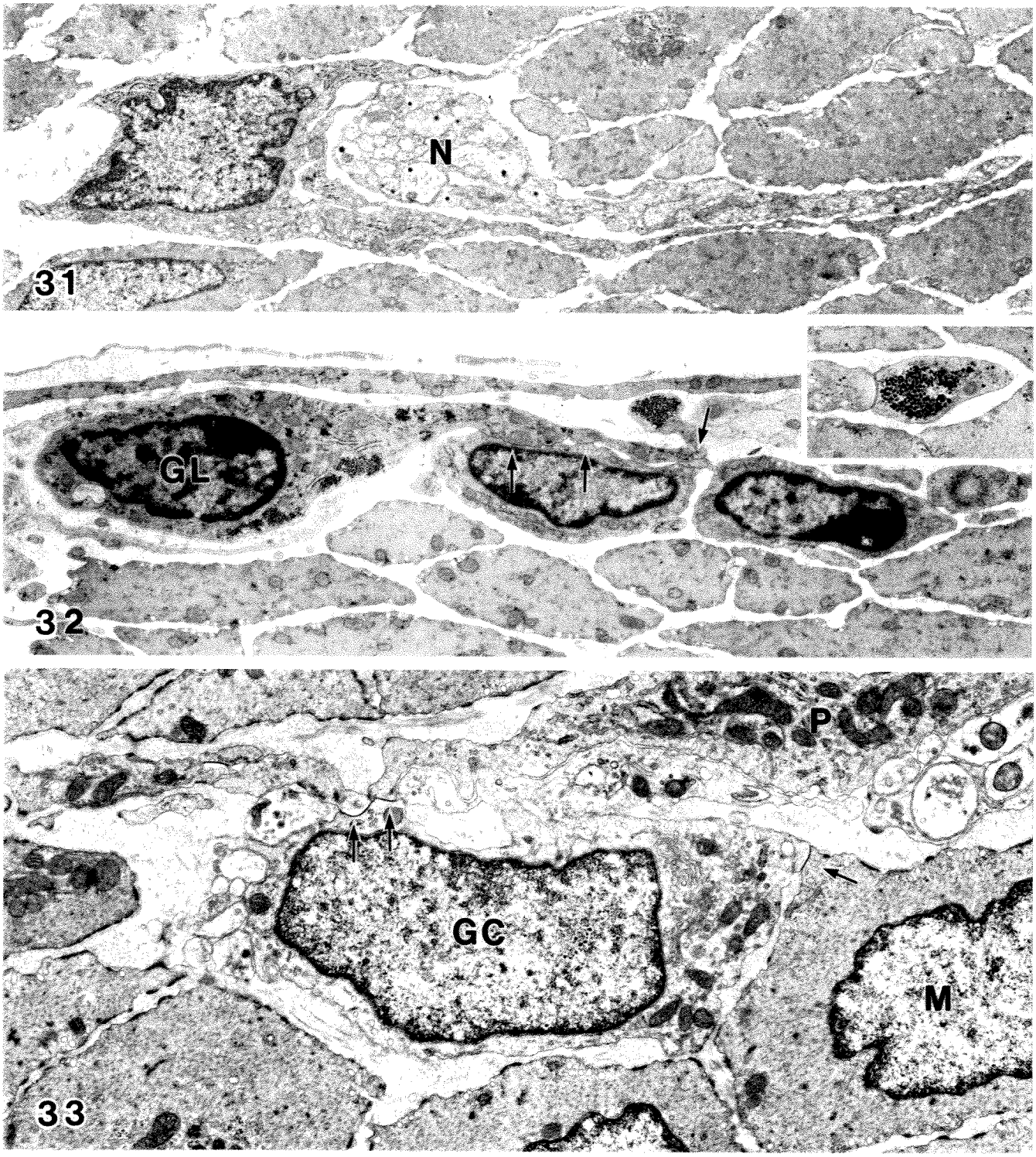

Fig. 31. A fibroblast-like interstitial cell located within the circular muscle layer of the guinea-pig small intestine. There is no basal lamina. N is a nerve bundle. x 9,000

Fig. 32. A glycogen-rich cell (GL) in the guinea-pig DMP region. Its gap junctions with an adjacent cell are indicated by arrows. N is a nerve bundle. x 10,000 **Insertion:** Higher magnification of the gap junction between the processes of glycogen-rich cells. x 18,000

Fig. 33. A gap junction-rich cell (GC) of DMP region of the rat small intestine. Arrows indicate gap junctions with the processes of the same type of cells (P) and with muscle cell (M). Numerous mitochondria are seen in the processes. Golgi apparatus (G) is located in the paranuclear region. x 12,000. From Komuro and Seki, 1995.

pattern of the branching processes, though they do show some resemblance to them.

ICC-DMP of the guinea-pig small intestine depicted with the Golgi method (Cajal, 1911, Fig. 575) may be different from those of the myenteric region and within the circular muscle layer observed by methylene blue staining. It puzzles us as to how to estimate the exact orientation of the cell bodies of ICC-DMP in the nerve network, though empty spaces in the midst of nerve bundles were indicated for the ICC (Cajal, 1911). ICC-DMP of the guinea-pig were apparently observed under a different staining condition from that used to visualize the whole nerve plexus. These cells may represent a cell type different from ICC-AP and ICC-CM which are stained together with nerves with methylene blue, since different staining effects often result from silver impregnation. This assumption is compatible with the observation that ICC-DMP is never stained by supravital methylene blue in the mouse, and ICC-DMP have not been successfully stained with this method in other species (Thuneberg, 1982).

The present observation indicates the existence of a group of cells which are not stained with the ZIO method, but which are closely associated with nerve bundles. The peculiar location of the cell bodies and size and shape of the nuclei may support the view that they constitute an independent cell type, rather than being simply unstained cells.

We also demonstrated that most of vimentin immunoreactive cells with numerous processes do not match to the figures of the ZIO-stained cells, but appear to correspond to the cells which are stained by Bauer's staining for glycogen. Since the main population of the ZIO-positive cells in DMP (i.e. the first type in the present study) are fibroblast-like cells (Zhou and Komuro, 1995), and since the gap junction-rich cells of the guinea-pig DMP do not to take a stellate form with many processes (Zhou and Komuro, 1992a), glycogen-rich cells (Fig. 32) among three types of interstitial cells in the guinea-pig DMP region (Zhou and Komuro, 1992a,b) are most likely to be ICC-DMP and correspond to the unstained cells with the ZIO method.

If this is the case, the ICC-DMP illustrated by Cajal may represent a unique type of cell, since glycogen-rich cells have not been observed in other species so far. However, it cannot be ruled out that the glycogen-rich cells instead of the gap junction-rich cells in the guinea-pig (Zhou and Komuro, 1992a,b) correspond to a certain population of the myoid cells which form many large gap junctions and are generally regarded as ICC in other species, including mouse (Yamamoto, 1977; Rumessen et al., 1982; Thuneberg, 1982), rat (Komuro and Seki, 1995; Fig. 33) and dog (Duchon et al., 1974; Torihashi et al., 1993). ICC associated with the DMP of human small intestine were reported to resemble smooth muscle cells and to form only occasional small gap junctions (Rumessen et al., 1992).

ICC-SMP

The ICC-SMP of the colon have been considered to correspond to ICC-DMP (Rumessen et al., 1982; Berezin et al., 1988). And the cells similar to those of the mouse DMP (Rumessen et al., 1982) were reported in the dog (Berezin et al., 1988; Torihashi et al., 1994) and in human (Rumessen et al., 1993b), though the gap junctions were not identified in the latter. Their functional implications for pacemaking activity have been well documented (Conklin and Du, 1990; Serio et al., 1990; Liu et al., 1994). However, it is not certain whether they really correspond to the ICC-DMP which were originally described in the guinea-pig small intestine (Cajal, 1911), because the latter cell type itself has not been clearly identified, for the reason just mentioned above.

Interstitial cells associated with guinea-pig SMP are only of one type, i.e., gap junction-rich cells (Ishikawa and Komuro, 1966), whereas three types of interstitial cells have been distinguished in the DMP of the same animal (Zhou and Komuro, 1992a,b). The former does not contain massive glycogen granules, even though the ICC-DMP of the guinea-pig are considered to be the glycogen-rich cells. The cells of SMP are stained with ZIO and vimentin antiserum (Ishikawa and Komuro, 1996).

ICC-LM

The cells of the longitudinal muscle layer show similar staining affinity to the cells of the circular muscle layer in the ZIO method. Although they are different from each other in appearance, it can be assumed that the same type of cells distribute throughout the external muscle coat take different cell shapes adapted to their tissue environment, such as the density of muscle bundles or the pattern of nerves that they associate with.

Interstitial cells of the serosa

Interstitial cells of the serosa in the present study were similar in the pattern of their cytoplasmic processes to those of the mouse stained with methylene blue (Thuneberg, 1982) and with the ZIO method (Rumessen and Thuneberg, 1982), and those of the guinea-pig demonstrated by scanning electron microscopy (Baluk and Gabella, 1987), as well as to cells of the rabbit small intestine stained with silver impregnation (Richardson, 1960). They showed very strong immunoreactivity to vimentin antiserum, and probably represent typical fibroblasts. In this context, it is a puzzling observation that the subserosal interstitial cells of the guinea-pig small intestine show strong cyclic GMP immunoreactivity after sodium nitroprusside stimulation (Young et al., 1993).

Functional role of ICC

The discussion in this article is basically confined to the morphological identification of ICC. However, the following brief description will refer to some functional aspects relating to the ICC.

c-kit receptor immunostaining reveals a cellular network which is independent from the myenteric plexus (present observation; Komuro and Zhou, 1996). It consists of characteristically shaped cells which have spindle or triangular cell bodies with slender cytoplasmic processes, showing a dichotomous branching pattern. They closely resemble cells depicted by ZIO method and vimentin immunostaining which we regarded as ICC-AP. Therefore, it can be concluded that ICC-AP correspond to *c-kit* receptor immunoreactive cells.

Since it has been suggested that the *c-kit* receptor is required for the normal development of pacemaker cells (Maeda et al., 1992; Torihashi et al., 1995), and that their defects result in a loss of slow waves (Ward et al., 1994; Huizinga et al., 1995) in the mouse intestine, it is very likely that at least ICC-AP have a pacemaker function. A recent study using *Ws/Ws* mutant rats (Isozaki et al., 1995) also indicated that abnormalities in the ileal movement and pyloric sphincter function are attributable to a deficiency of *c-kit* mRNA-expressing cells.

On the other hand, ICC-DMP probably play a role in the impulse conduction system, as suggested by (Thuneberg, 1982), because they form many gap junctions with one another and with smooth muscle cells in addition to their close contacts with nerve terminals, though no direct physiological evidence has been reported so far.

As to the function of ICC-CM, Thuneberg (1989) reported that their conductive function is questionable, since he had found only intermediate-type junctions with each other and with smooth muscle cells. However, gap junctions have been detected between ICC-CM and smooth muscle cells of the rabbit colon (Komuro, 1982) and of the guinea-pig small intestine (Zhou and Komuro, 1992b). Thus, ICC-CM are quite likely to have a conductive function. Indeed, it was in the gizzard of the love-bird in which the gap junctions between the interstitial cells and smooth muscle cells were found for the first time (Imaizumi and Hama, 1969).

Concluding remarks

The present study clearly demonstrates the real existence of particular types of cells which show exactly the same characteristics as the ICC depicted in the original drawings (Cajal, 1911). It also reveals that ICC described in the myenteric region, DMP and within the circular muscle layer (Cajal, 1911) are ultrastructurally heterogeneous and correspond to the different type of cells in each location.

Regarding the cell nature, Prosser et al. (1989) reported that ICC of the rat small intestine appear to be some type of neuron, because they are stained with neuron specific enolase (NSE) antiserum but are devoid of immunoreactivity for four types of intermediate filaments; GFAP, vimentin, desmin and neurofilament. However, their immunoreactivity seems not to localize to ICC, and no cells immunopositive to NSE were detected in the myenteric and DMP regions of the guinea-pig intestine (Tokui et al., 1992; Zhou and Komuro, 1992a).

ICC-AP, ICC-CM and ICC-LM as well as the interstitial cells of the serosa are probably categorized into fibroblast-like cells. Although ICC-AP with some myoid features were reported in the mouse (Thuneberg, 1982), dog (Berezin et al., 1990; Xue et al., 1993) and human (Faussonne-Pellegrini et al., 1990; Rumessen and Thuneberg, 1991), the present observation clearly demonstrates that ICC-AP and ICC-CM being identical with the original drawings, are fibroblast-like cells. It should be emphasized here that cells with myoid features such as basal lamina and many caveolae have never been identified in the myenteric region of the rat (Komuro, 1989) or the guinea-pig (Zhou and Komuro, 1995) small intestine. A cell type resembling the typical fibroblast with small gap junctions was further observed in the myenteric region of the rabbit colon (Komuro, 1982). Thus, if all these species really have equivalent cell types in corresponding locations, those ICC-AP with a different appearance can be considered as species variations of the fibroblast-like cells rather than those of smooth muscle cells, since it has been well documented that a family of fibroblasts shows a wide diversity of cytological features and that differences among these cells are quantitative rather than qualitative (Komuro, 1990).

Those cells probably represent a subtype of fibroblasts which are specialized for intercellular communication (and/or pacemaker function) in the smooth muscle tissue, and display a different degree of fibroblastic-myoid features depending on their microenvironment, as determined by tissue layer, or level of the alimentary tract, or species.

On the other hand, since some myoid features were shown in every putative ICC-DMP from different species, including mouse (Yamamoto, 1977; Rumessen et al., 1982; Thuneberg, 1982), rat (Komuro and Seki, 1995), guinea-pig (Zhou and Komuro, 1992a,b), dog (Duchon et al., 1974; Torihashi et al., 1993) and human (Rumessen et al., 1992), it is very likely that ICC-DMP belong to a special type of smooth muscle cells. Furthermore, ICC-DMP show more myoid features, including dense bodies and better developed basal lamina, as well as caveolae and subsurface cisterns in species with a larger body size (dog, human) than those with smaller body size (mouse, rat, guinea-pig).

Novotny and Gnoth (1991) demonstrated considerable differences in size and morphology of fibroblasts in the human digital dermis and subcutis by using silver impregnation. Further, Wake and Sato (1993) reported that the perisinusoidal stellate cells (fat-

storing cells) of the porcine liver display marked morphological heterogeneity in their location in the hepatic lobule, using the Golgi method. These observations may explain why the ZIO method, which is generally regarded as a specific staining method for nervous elements, revealed a wide variety of interstitial cells in different tissue layers including typical serosal fibroblasts in the present study.

It is an important issue for future studies to characterize the functional significance of ICC in each location.

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References

Anderson C.R. and Edwards S.L. (1993). Subunit b of cholera toxin labels interstitial cells of Cajal in the gut of rat and mouse. Histochemistry 100, 457-464.

Baluk P. and Gabella G. (1987). Scanning electron microscopy of the muscle coat of the guinea-pig small intestine. Cell Tissue Res. 250, 551-561.

Berezin I., Huizinga J.D. and Daniel E.E. (1988). Interstitial cells of Cajal in the canine colon: a special communication network at the inner border of the circular muscle. J. Comp. Neurol. 273, 42-51.

Berezin I., Huizinga J.D. and Daniel E.E. (1990). Structural characterization of interstitial cells of Cajal in myenteric plexus and muscle layers of canine colon. Can. J. Physiol. Pharmacol. 68, 1419-1431.

Bjorklund H., Dahl D. and Seiger A. (1984). Neurofilament and glial fibrillary acid protein-related immunoreactivity in rodent enteric nervous system. Neuroscience 12, 277-287.

Boeke J. (1949). The sympathetic end formation, its synaptology, the interstitial cells, the periterminal network, and its bearing on the neurone theory. Discussion and critique. Acta Anat. 8, 18-61.

Cajal S.R. (1893). Sur les ganglions et plexus nerveux de l'intestin. Compt. Rend. Soc. Biol. Paris 45, 217-223.

Cajal S.R. (1911). Histologie du systeme nerveux de l'homme et des vertebres. Tome 2. Maloine. Paris.

Christensen J. (1992). A commentary on the morphological identification of interstitial cells of Cajal in the gut. J. Auton. Nerv. Syst. 37, 75-88.

Christensen J., Rick G.A. and Soll D.J. (1987). Intramural nerves and interstitial cells revealed by the Champy-Maillet stain in the opossum esophagus. J. Auto. Nerv. Syst. 19, 137-151.

Conklin J.L. and Du C. (1990). Pathways of slow-wave propagation in proximal colon of cats. Am. J. Physiol. 258 (Gastrointest. Liver Physiol. 21). G894-G903.

Du C. and Conklin J.L. (1989). Origin of slow waves in the isolated proximal colon of the cat. J. Auton. Nerv. Syst. 28, 167-178.

Duchon G., Henderson R. and Daniel E.E. (1974). Circular muscle layers in the small intestine. In: Proc. 4th Int. Symp. Gastrointest. Daniel E.E. (eds). Motil. Banff, Alberta, Canada. Mitchell Press. Vancouver. pp 635-646.

Faussone-Pellegrini M.S. (1984). Morphogenesis of the special circular muscle layer and of the interstitial cells of Cajal related to the plexus muscularis profundus of mouse intestinal muscle coat. An EM study. Anat. Embryol. 169, 151-158.

Faussone-Pellegrini M.S. (1985). Cytodifferentiation of the interstitial cells of Cajal related to the myenteric plexus of mouse intestinal muscle coat. An EM study from fetal to adult life. Anat. Embryol. 171, 163-169.

Faussone-Pellegrini M.S. (1987). Cytodifferentiation of the interstitial cells of Cajal of mouse colonic circular muscle layer: An EM study from fetal to adult life. Acta Anat. 128, 98-109.

Faussone-Pellegrini M.S., Pantalone D. and Cortesini C. (1989). An ultrastructural study of the interstitial cells of Cajal of the human stomach. J. Submicrosc. Cytol. Pathol. 21, 439-460.

Faussone-Pellegrini M.S., Cortesini C. and Panetalone D. (1990). Neuromuscular structures specific to the submucosal border of the human colonic circular muscle layer. Can. J. Physiol. Pharmacol. 68, 1437-1446.

Hara Y., Kubota M. and Surszerszewski J.H. (1986). Electrophysiology of smooth muscle of the small intestine of some mammals. J. Physiol. 372, 501-520.

Huizinga J.D., Shin A. and Chow E. (1988). Electrical coupling and pacemaker activity in colonic smooth muscle. Am. J. Physiol. 255 (Cell Physiol. 24), C653-C660.

Huizinga J.D., Thuneberg L., Kluppel M., Malysz J., Mikkelsen H.B. and Berstein A. (1995). W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature 373, 341-349.

Imaizumi M. and Hama K. (1969). An electron microscopic study on the interstitial cells of the gizzard in the love-bird (*Uroloncha domestica*). Z. Zellforsch. 97, 351-357.

Ishikawa K. and Komuro T. (1996). Characterization of the interstitial cells associated with the submuscular plexus of the guinea-pig colon. Anat. Embryol. (in press).

Isozaki K., Hirota S., Nakama A., Miyagawa J., Shinomura Y., Xu Z.D., Nomura S. and Kitamura Y. (1995). Distributed intestinal movement, bile reflux to the stomach, and deficiency of c-kit-expressing cells in Ws/Ws mutant rats. Gastroenterology 109, 456-463.

Jessen K.R. and Mirsky R. (1980). Glial cells in the enteric nervous system contain glial fibrillary acidic protein. Nature 286, 736-737.

Jessen K.R. and Mirsky R. (1985). Glial fibrillary acidic polypeptides in peripheral glia. Molecular weight, heterogeneity and distribution. J. Neuroimmunol. 8, 377-393.

Jessen H. and Thuneberg L. (1991). Interstitial cells of Cajal and Auerbach's plexus. A scanning electron microscopical study of guinea-pig small intestine. J. Submicrosc. Cytol. Pathol. 23, 195-212.

Karnovsky M.J. and Roots L. (1964). A direct-coloring thiocholine method for cholinesterases. J. Histochem. Cytochem. 12, 219-221.

Keith A. (1914/1915). An account of six specimens fo the great bowel removed by operation: with some observations on the motor mechanism of the colon. Br. J. Surg. 2, 576-599.

Keith A. (1915). A new theory of the causation of enterostasis. Lancet 2, 371-375.

Keith A. (1916). Proceedings of the Anatomical Society of Great Britain and Ireland. J. Anat. Physiol. 50, 1-3.

Kobayashi S., Suzuki M., Endo T., Tsuji S. and Daniel E.E. (1986). Framework of the enteric nerve plexuses: an immunocytochemical study in the guinea pig jejunum using and antiserum to S-100 protein. Arch. Histol. Jpn. 49, 159-188.

Kobayashi S., Furness J.B., Smith T.K. and Pompolo S. (1989). Histological identification of the interstitial cells of Cajal in the guinea-pig small intestine. Arch. Histol. Cytol. 52, 267-286.

Komuro T. (1982). The interstitial cells in the colon of the rabbit: scanning and transmission electron microscopy. Cell Tissue Res. 222, 41-51.

Komuro T. (1987). Three-dimensional structure of the intestinal wall. Thera Res. 7, 107-115.

Komuro T. (1989). Three-dimensional observation of the fibroblast-like cells associated with the rat myenteric plexus, with special reference to the interstitial cells of Cajal. Cell Tissue Res. 255, 343-351.

Komuro T. (1990). Re-evaluation of fibroblasts-like and fibroblast cells. Anat. Embryol. 182, 103-112.

Komuro T. and Seki K. (1995). Fine structural study of interstitial cells associated with the deep muscular plexus of the rat small intestine, with special reference to the intestinal pacemaker cells. Cell Tissue Res. 282, 129-134.

Komuro T. and Zhou D.S. (1996). Anti-c-kit protein immunoreactive cells corresponding to the interstitial cells of Cajal in the guinea-pig small intestine. J. Auto. Nerv. Syst. (in press).

Komuro T., Zhou D.S. and Tokui K. (1994). Identification of the interstitial cells of Cajal. XIV Federative International Congress of Anatomy. Lisbon, p 169. (Abstracts).

Liu L.W.C., Thuneberg L., Daniel E.E. and Huizinga J.D. (1993). Selective accumulation of methylene blue by interstitial cells of Cajal in canine colon. Am. J. Physiol. 264 (Gastrointest. Liver Physiol. 27), G64-G73.

Liu L.W.C., Thuneberg L. and Huizinga J.D. (1994). Selective lesioning of interstitial cells of Cajal by methylene blue and light leads to loss of slow waves. Am. J. Physiol. 266 (Gastrointest. Liver Physiol. 29), G4854-G496.

Maeda H., Yamagata A., Nishikawa S., Yoshinaga K., Kobayashi S., Nishi K. and Nishikawa S. (1992). Requirement of c-kit for development of intestinal pacemaker system. Development 116, 369-375.

Meyling H.A. (1953). Structure and significance of the peripheral extension of the autonomic nervous system. J. Comp. Neurol. 99, 495-543.

Nada O. and Kawana T. (1988). Immunohistochemical identification of supportive cell types in the enteric nervous system of the rat colon and rectum. Cell Tissue Res. 251, 523-529.

Novotny G.E.K. and Gnoth C. (1991). Variability of fibroblast morphology *in vivo*: a silver impregnation study on human digital dermis and subcutis. J. Anat. 177, 195-207.

Prosser C.L., Holzwarth M.A. and Barr L. (1989). Immunocytochemistry of the interstitial cells of Cajal in the rat intestine. J. Auton. Nerv. Syst. 27, 17-25.

Richardson K.C. (1958). Electron microscopic observations on Auerbach's plexus in the rabbit, with special reference to the problem of smooth muscle innervation. Am. J. Anat. 103, 99-135.

Richardson K.C. (1960). Studies on the structure of autonomic nerves in the small intestine, correlating the silver-impregnated image in light microscopy with the permanganate-fixed ultrastructure in electron microscopy. J. Anat. 94, 457-472.

Rumessen J.J. and Thuneberg L. (1982). Plexus muscularis profunds and associated interstitial cells. I. Light microscopical studies of mouse small intestine. Anat. Rec. 203, 115-127.

Rumessen J.J. and Thuneberg L. (1991). Interstitial cells of Cajal in human small itnestine. Ultrastructural identification and organization between the main smooth muscle layers. Gastroenterology 100, 1417-1431.

Rumessen J.J., Thuneberg L. and Mikkelsen H.B. (1982). Plexus muscularis profunds and associated interstitial cells. II. Ultrastructural studies of mouse small intestine. Anat. Rec. 203, 129-146.

Rumessen J.J., Mikkelsen H.B. and Thuneberg L. (1992). Ultrastructure of interstitial cells of Cajal associated with deep muscular plexus of human small intestine. Gastroenterology 102, 56-88.

Rumessen J.J., Mikkelsen H.B., Qvortrup K. and Thuneberg L. (1993a). Ultrastructure of interstitial cells of Cajal in circular muscle of human small intestine. Gastroenterology 104, 343-350.

Rumessen J.J., Peters S. and Thuneberg L. (1993b). Light and electron microscopical studies of interstitial cells of Cajal and muscle cells at the submucosal border of human colon. Lab. Invest. 68, 481-495.

Serio R., Huizinga J.D., Barajas-Lopez C. and Daniel E.E. (1990). Interstitial cells of Cajal and slow wave generation in canine colonic circular muscle. J. Auton. Nerv. Syst. 30, S141-S144.

Shuttleworth C.W., Xue C., Ward S.M., De Vente J. and Sanders K.M. (1993). Immunohistochemical localization of 3',5'-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. Neuroscience 56, 513-522.

Suzuki M., Prosser C.L. and Dahms V. (1986). Boundary cells between longitudinal and circular layers: essential for electrical slow waves in cat intestine. Am. J. Physiol. 250, G287-G294.

Taxi J. (1965). Contribution a l'etude des connexions des neurones moteurs du systeme nervoux autonome. Ann. Sci. Nat. Zool. Tome VII, pp 413-474.

Thuneberg L. (1982). Interstitial cells of Cajal: intestinal pacemaker cells? Adv. Anat. Embryol. Cell. Biol. 71, 1-30.

Thuneberg L. (1989). Interstitial cells of Cajal. In: Handbook of physiology. The gastrointestinal system. Vol. 1. Woods J.D. (ed.) American Physiology Society. Bethesda, Maryland. pp 349-386.

Thuneberg L., Rumessen J.J. Mikkelsen H.B., Peters S. and Jessen H. (1995). Structural aspects of interstitial cells of Cajal as intestinal pacemaker cells. In: Pacemaker activity and intercellular communication. Huizinga J.D. (ed.). CRC Press. Boca Raton. pp 193-222.

Tokui K., Zhou D.S. and Komuro T. (1992). Immunohistochemical study of the interstitial cells of Cajal. J. Smooth Muscle Res. 28, 380-382.

Torihashi S., Kobayashi S., Gerthoffer W.T. and Sanders K.M. (1993). interstitial cells in deep muscular plexus of canine small intestine may be specialized smooth muscle cells. Am. J. Physiol. 265, G638-G645.

Torihashi S., Gerthoffer W.T., Kobayashi S. and Sanders K.M. (1994). Identification and classification of interstitial cells in the canine proximal colon by ultrastructure and immunohistochemistry. Histochemistry 101, 169-183.

Torihashi S., Ward S.M., Nishikawa S., Nish K., Kobayashi S. and Sanders K.M. (1995). *c-kit* dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res. 280, 97-111.

Vajda J. and Feher E. (1980). Distribution and fine structure of the interstitial cells of Cajal. Acta Morphol. Acad. Sci. Hung. 28, 251-258.

Wake K. and Sato T. (1993). Intralobular heterogeneity of perisinusoidal stellate cells in porcine liver. Cell Tissue Res. 273, 227-237.

Ward S.M., Burns A.J., Torihashi S. and Sanders K.M. (1994). Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. J. Physiol. (London) 480, 91-97.

Xue C., Ward S.M., Shuttleworth W. and Sanders K.M. (1993). Identification of interstitial cells in canine proximal colon suing NADH diaphorase histochemistry. Histochemistry 99, 373-384.

Xue C., Pollock J., Schmidt H.H.H.W., Ward S.M. and Sanders K.M. (1994). Expression of nitric oxide synthase immunoreactivity by interstitial cells of the canine proximal colon. J. Auto. Nerv. Syst. 49, 1-14.

Yamamoto M. (1977). Electron microscopic studies on the innervation of the smooth muscle and the interstitial cell of Cajal in the small intestine of the mouse and bat. Arch. Histol. Jpn. 40, 171-201.

Young H.M., McConalogue K., Furness J.B. and De Vente J. (1993). Nitric oxide targets in the guinea pig intestine identified by induction of cyclic GMP immunoreactivity. Neuroscience 56, 513-522.

Zhou D.S. and Komuro T. (1992a). Interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine, with special reference to the interstitial cells of Cajal. Cell Tissue Res. 268, 205-216.

Zhou D.S. and Komuro T. (1992b). The cellular network of interstitial cells associated with the deep muscular plexus of the guinea pig small intestine. Anat. Embryol. 186, 519-527.

Zhou D.S. and Komuro T. (1995). Ultrastructure of the zinc iodide-osmic acid stained cells in guinea pig small intestine. J. Anat. 187, 481-485.

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ORIGINAL ARTICLE

Koichi Ishikawa · Terumasa Komuro

Characterization of the interstitial cells associated with the submuscular plexus of the guinea-pig colon

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Abstract Interstitial cells associated with the submuscular plexus of the guinea pig colon were studied by electron microscopy and by light microscopic whole-mount stretch preparations. Their cytoplasmic features are similar to those of fibroblasts and they contain a well-developed Golgi apparatus, granular endoplasmic reticulum and many mitochondria. Intermediate filaments are abundantly distributed throughout the perinuclear region and processes. Numerous caveolae, a basal lamina and subsurface cisterns are observed on the cell membrane as in smooth muscle cells. The most characteristic feature of this cell type is the existence of many large gap junctions that interconnect these cells to each other and with the smooth muscle cells. Nerve varicosities containing synaptic vesicles are observed in close apposition with cells of this type. Whole-mount preparations stained by the zinc iodide-osmic acid method and by vimentin immunohistochemistry clearly demonstrated the stellate form of these gap junction-rich cells and suggested that they correspond to the interstitial cells of Cajal.

Key words Pacemaker · Interstitial cells of Cajal · Intestine · Ultrastructure · Immunohistochemistry

Introduction

Interstitial cells of Cajal (ICC; 1893, 1911) have become central to the understanding of gastro-intestinal motility since their putative function as pacemakers and the impulse-conductive role of the intestinal musculature were proposed (Thuneberg 1982). Berezin et al. (1988, 1990) observed interstitial cells at the circular muscle-submucosa interface of the canine colon and pointed out their morphological similarities to ICC-III, described in the deep muscular plexus (DMP) of the mouse small intestine (Rumessen et al. 1982; Thuneberg 1982). They suggested that a complex of nerves, ICC and muscle cells, connected by gap junctions, might provide a structural basis for the pacemaker function of the canine colonic musculature.

In fact, electrical slow waves, representing physiological signals of pacemaker activities, are recorded at the submucosal border of the circular muscle layer containing ICC of dog colon (Serio et al. 1991; Ward et al. 1991; Liu et al. 1992) and cat colon (Conklin and Du 1990; Du and Conklin 1989).

However, a wide variety of interstitial cells is reported as ICC at different locations in the gastrointestinal tract in different species (Thuneberg 1989; Christensen 1992), and the morphological identification of ICC remains controversial. Although ICC associated with the submuscular plexus (SMP) of the colon have been studied in the rat (Stach 1972), the mouse (Faussone-Pellegrini 1985), the dog (Berezin et al. 1988; Xue et al. 1993; Torihashi et al. 1994) and humans (Faussone-Pellegrini et al. 1990; Rumessen et al. 1993), it has not been fully understood whether morphological discrepancies among these observations indicate species differences or not. For example, one of the important ultrastructural features, the gap junctions, has not been observed in the human specimens (Rumessen et al. 1993).

The present study intends to characterize interstitial cells closely associated with the SMP of the guinea-pig proximal colon by electron microscopy, since they have not been clearly described in spite of the fact that ICC of the DMP, presumed as an equivalent for the SMP, were originally described in this animal (Cajal 1911). We also correlate light-microscopic observations to electron-microscopic observations by reprocessing the whole mount preparations stained by the zinc iodide-osmic acid (ZIO) method and by vimentin-immunohistochemistry. This approach may provide an important clue to correlate ultrastructural features of a given cell type to ICC, depicted by methylene-blue staining or by the Golgi method, and to discern the developmental origin of the interstitial cells.

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Materials and methods

Transmission electron microscopy

Pieces of guinea-pig proximal colon were placed in a fixative containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 3 h at 4° C. The specimens were then rinsed in the same buffer and post-fixed in 1% osmium tetroxide in the same buffer for 2 h at 4° C. Following osmication, the specimens were rinsed in distilled water, block-stained with a saturated aqueous uranyl acetate solution for 2 h, dehydrated in a graded series of ethyl alcohols, and embedded in Epon epoxy resin. Ultrathin sections were cut using a Reichert ultramicrotome and double-stained with uranyl acetate and lead tartrate for observation under a JEOL JEM 1200EX II electron microscope.

Light microscopy

Zinc iodide-osmic acid (ZIO) impregnation

Short segments of guinea-pig proximal colon were moderately inflated and fixed for 24 h at room temperature with fixative containing 0.4% O_SO_4 and 2.4% ZnI_2. The specimens were then rinsed in distilled water and cut along the mesentery to make flat sheets. Under a dissecting microscope, circular muscle layers were carefully laminated from the mucosa and from the longitudinal muscle layers with fine forceps. The isolated circular muscle layers were distended and mounted with aqueous mounting medium "Mount-quick (Daido Sangyo)" for observation under an Olympus BH2 light microscope. For transmission electron microscopy, suitable areas of the specimens were cut off, block-stained with a saturated aqueous uranyl solution for 2 h and then processed for electron microscopy as mentioned above.

Immunohistochemistry for vimentin

Short segments of guinea-pig proximal colon were inflated and fixed for 2 h at 4° C with fixative containing 2% paraformadehyde in 0.1 M phosphate buffer, pH 7.4. After rinsing in phosphate-buffered saline (PBS), the circular muscle layer was laminated as described above. Segments of the circular muscle layers were placed in PBS containing 0.3% Triton X-100 and 0.1% NaN_3, at 4° C for 1 h. The specimens were then stretched on glass slides and were incubated with the monoclonal antibody against vimentin (DAKO-Vimentin, V9, Code No. M 725) at a dilution ratio of 1:20. The primary antibody was visualized by the conventional immunoperoxidase method, using a DAB Substrate kit (Zymed Laboratories).

The specimens were mounted with Mount-quick and photographed with an Olympus light microscope. For transmission electron microscopy, selected areas of the specimens were cut off and processed for electron microscopic observations, omitting double-staining with uranyl acetate and lead tartrate so as not to obscure immunoreactive products.

Results

The circular muscle layer of the guinea-pig proximal colon is composed of about 40–50 layers of smooth muscle cells and is divided indistinctly by connective tissue septa that contain thick nerve bundles and blood vessels (Fig. 1). The SMP is located at the circular muscle-submucosa interface, and the nerve bundles mainly run parallel to the axis of the circular muscle cells. Fibroblasts and macrophages as well as Schwann cells are observed in association with these nerve bundles.

Among them, the most important structure is a special type of interstitial cell that is characterized by many large gap junctions interconnecting each other and neighboring smooth muscle cells (Figs. 2–4). The interconnecting junctions between interstial cells (Fig. 3) are usually larger in size and more frequent than the gap junctions between the interstitial cells and the smooth muscle cells (Fig. 4). The cell bodies of these gap junction-rich cells orientate almost parallel to the circular muscle cells and project many cytoplasmic processes extending in all directions. They are closely associated with nerve bundles, but are easily distinguished from Schwann cells that occupy a central position within the nerve bundles and hold nerve fibers by many radial processes.

The nuclei of the gap junction-rich cells are usually oval, with smooth contours. The cytoplasm has an electron density similar to that of adjacent muscle cells. In the perinuclear regions, there are many mitochondria and a well-developed Golgi apparatus (Fig. 2). The perinuclear cytoplasm also contains rough endoplasmic reticulum (RER) and free ribosomes. A basal lamina and many caveolae are observed on the surface of the cell membrane (Fig. 5). Bundles of intermediate filaments and thin filaments are seen throughout the cytoplasm (Fig. 6), but thick filaments and dense bodies have not been observed. Cilia or basal bodies are often encountered. Lipid droplets are rarely seen.

Although the cell bodies of these gap junction-rich cells can not be found deeper than a few muscle-cell layers, their processes penetrate into the innermost 2 or 3 muscle layers and form gap junctions with smooth muscle cells at that location (Fig. 7). Besides gap junctions, peg- and socket-like structures are also observed between this type of cell and smooth muscle cells (Fig. 8). These structures may serve as mechanical links.

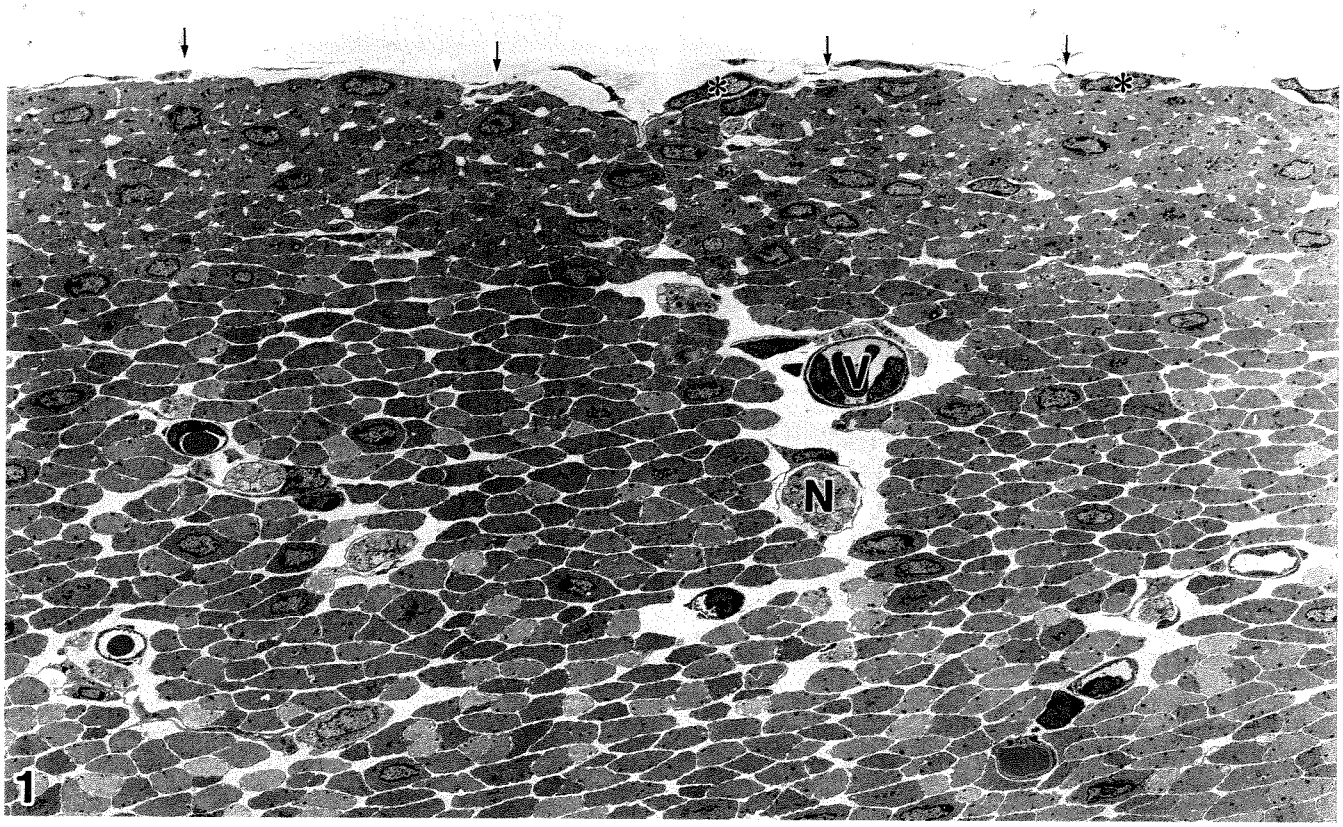
Nerve varicosities containing synaptic vesicles are often observed in close association (about 30–50 nm) with both the cell bodies and cell processes (Fig. 9), though no membrane specializations are detected on either side. These nerve varicosities appear to be classifiable into three types by the principal type of synaptic vesicle they

Fig. 1 Electron micrograph showing a cross section through the circular muscle layer of the guinea pig proximal colon. Blood vessels (*V*) and large nerve fibers (*N*) are seen in the connective tissue septum. Small nerve fibers (*arrows*) of the submuscular plexus and interstitial cells (*) are observed at the circular muscle-submucosa interface. This type of interstitial cell is not observed in the interior of the muscle layer. ×2,000

Fig. 2 Profile of a gap junction-rich cell (*GC*). Mitochondria, rough endoplasmic reticulum and well-developed Golgi apparatus can be seen in the perinuclear region. The gap junctions connecting with the same type of cell and the innermost smooth muscle cell are indicated by the *arrow* and the *arrowhead*, respectively. ×20,000

Fig. 3 Gap junction between two GC processes. ×150,000

Fig. 4 Gap junction between a GC (*GC*) and a smooth muscle cell (*SM*), smaller than the GC interconnecting gap junctions. ×80,000


V
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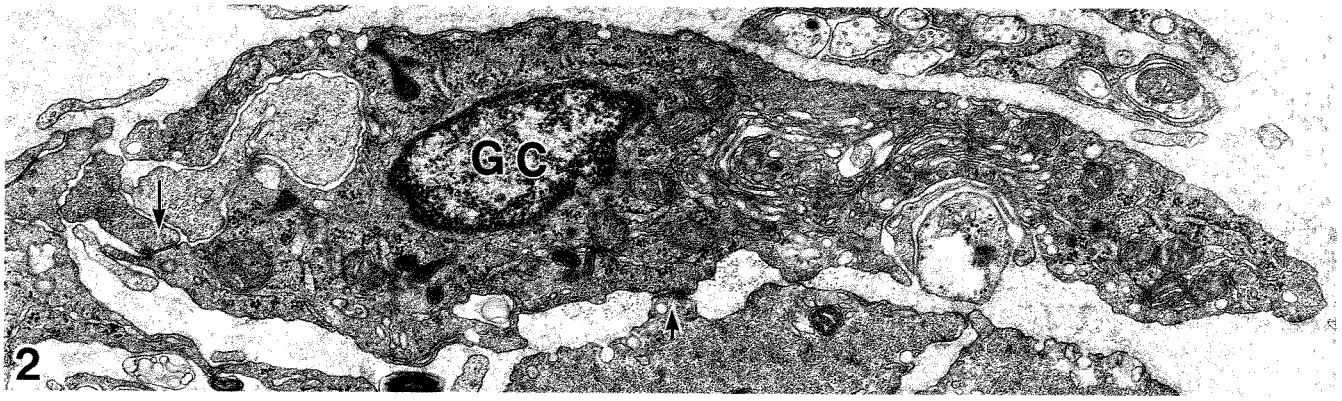
1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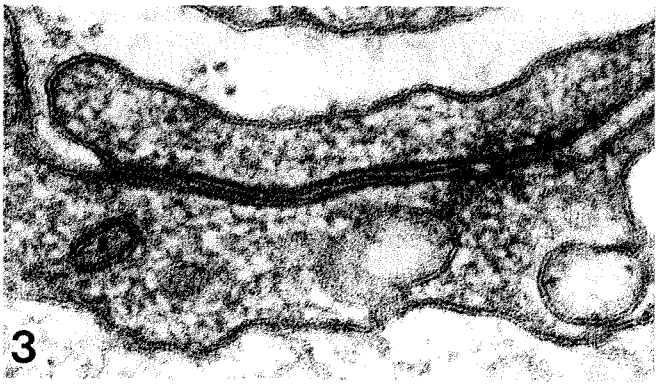
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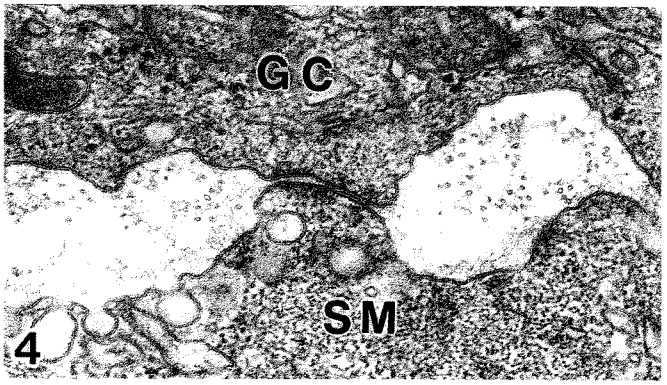
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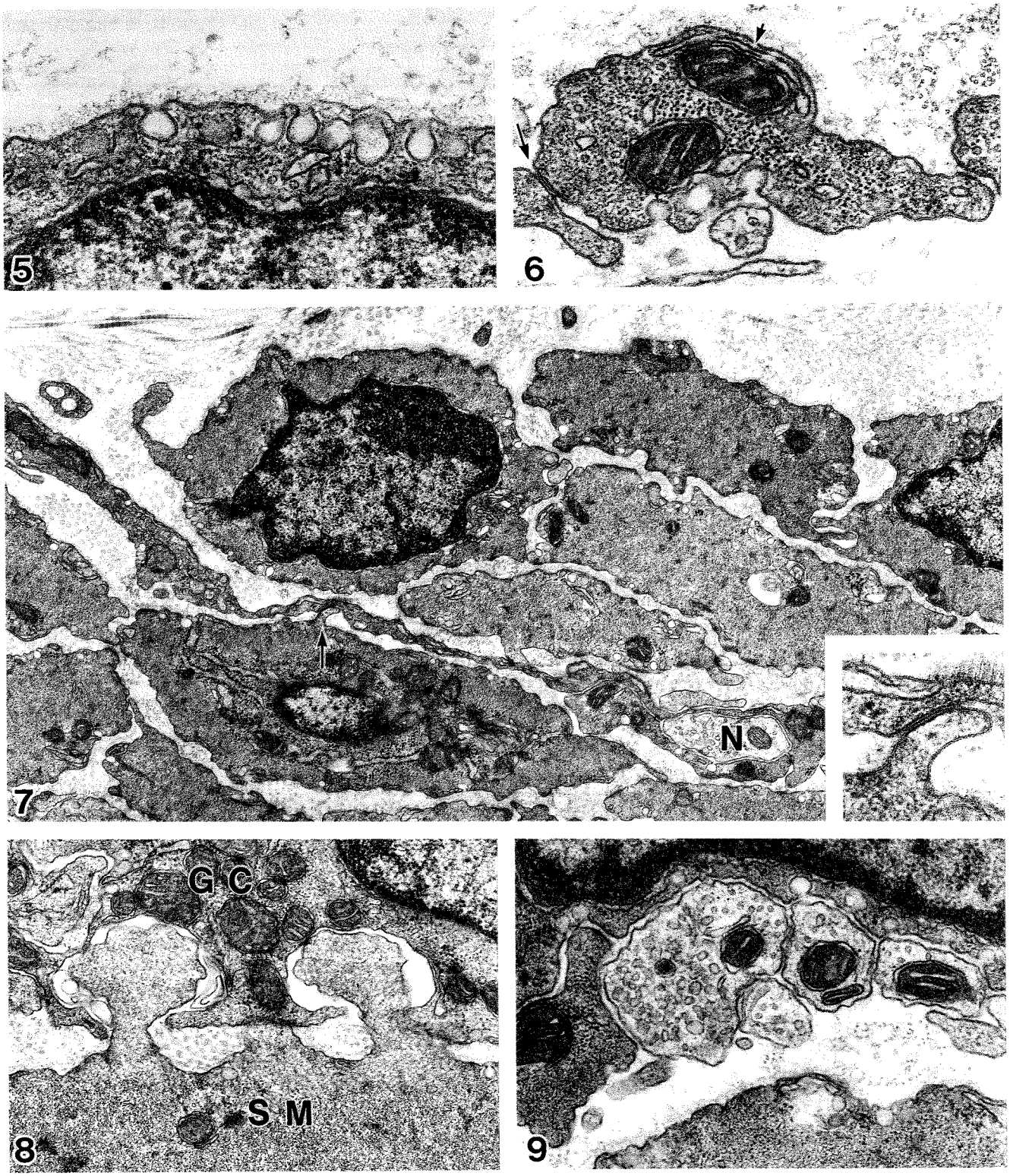


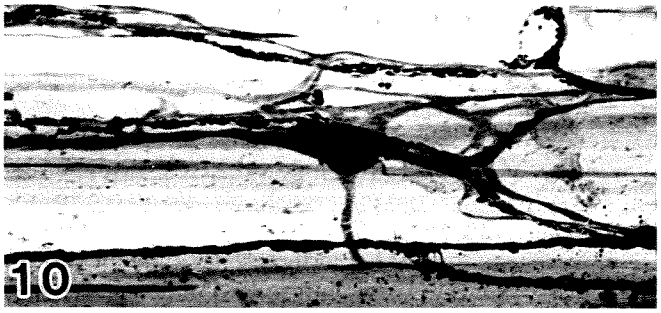
Fig. 5 Numerous caveolae and a basal lamina are observed on the cell surface. ×52,000

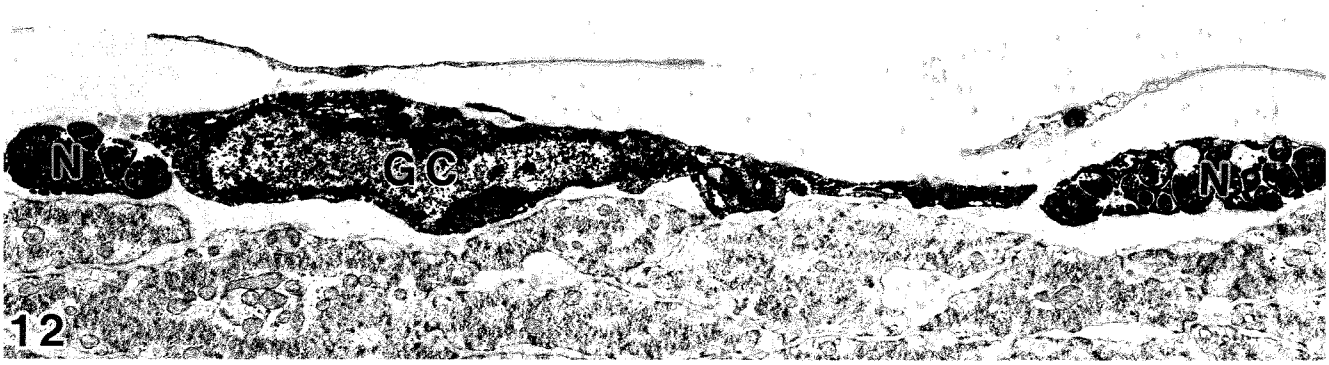
Fig. 6 Cross section of GC processes. Abundant intermediate filaments are observed in the cytoplasm. The *arrow* indicates a gap junction between these processes. The *arrowhead* indicates a structure resembling a subsurface cistern. ×60,000

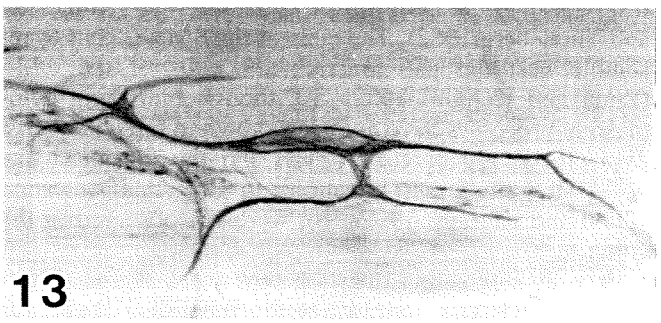
Fig. 7 Cytoplasmic processes extending from a GC to form a gap junction (*arrow*) with a smooth muscle cell at a depth of a few cell layers and a close contact with a nerve bundle (*N*). ×16,000. *Inset* Higher magnification to show the gap junction between the GC process and the smooth muscle cell. ×100,000

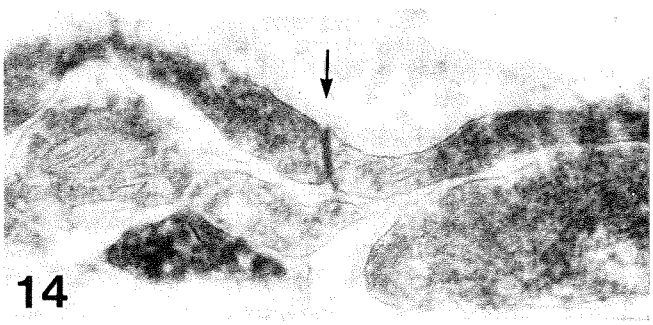
Fig. 8 Peg and socket-like structures between the GC (*GC*) and an adjoining smooth muscle cell (*SM*). ×12,000

Fig. 9 Nerve varicosities containing both small clear (about 50 nm) and large cored vesicles (about 100 nm) in close association (about 30–50 nm) with GC. ×36,000









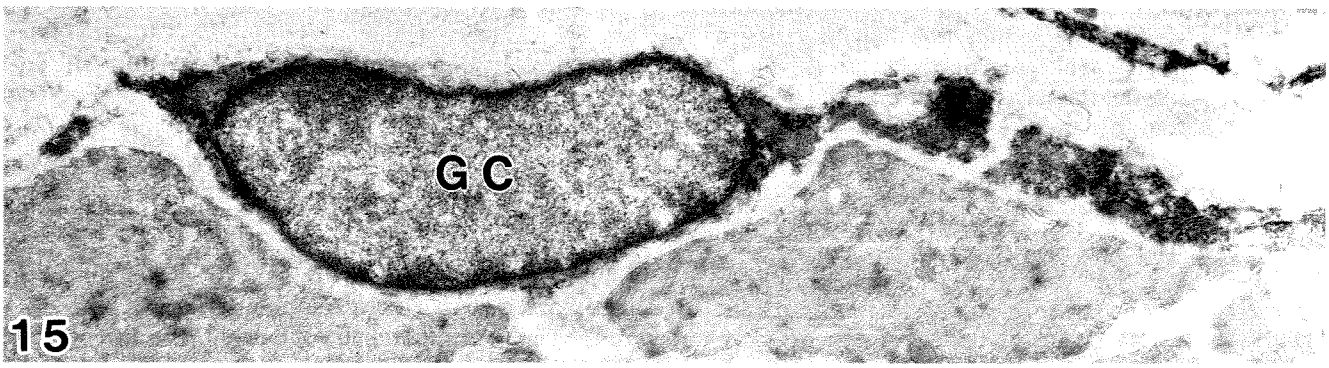


Fig. 10 Whole-mount stretch preparation of the guinea-pig proximal colon stained by the ZIO method. A stellate cell is observed beside the nerve fibers. ×700

Fig. 11 Gap junction between two GC processes stained with ZIO (*arrow*). ×60,000

Fig. 12 An electron micrograph showing a GC (*GC*) stained with ZIO beside the nerve fibers (*N*). ×8,000

Fig. 13 Stellate cell of the guinea pig proximal colon demonstrated with immunohistochemical staining for vimentin. ×1,200

Fig. 14 Gap junction between GC processes (*arrow*) that show immunoreactivity for vimentin. ×50,000

Fig. 15 Electron micrograph showing a vimentin-immuno-positive cell (*GC*) located at the circular muscle-submucosa interface. ×15,000

contain: small clear round vesicles, small clear flattened vesicles and large cored vesicles.

These gap junction-rich cells occasionally come into close contact with typical fibroblasts, but gap junctions have not been observed so far.

ZIO impregnation

On whole-mount preparations stained by the ZIO method, the SMP shows a polygonal meshwork consisting of nerve bundles oriented parallel to the circular muscle and transverse interconnecting fibers (Fig. 10). Stellate or fusiform cells with a slightly lighter shade than nerves are visualized beside these nerve bundles. Under the electron microscope, the ZIO-stained cells are easily identified by their electron-dense deposits caused by the ZIO-mixture at the circular muscle-submucosa interface (Fig. 12). Although their fine structures are often obscured by dense deposits, their identification as gap junction-rich cells is not difficult because of their large gap junctions and their peculiar location at the muscle-submucosa interface (Fig. 11).

Immunohistochemistry for vimentin

Stellate cells resembling ZIO-stained cells are clearly observed on whole-mount stretch preparations stained with vimentin-antiserum, though their relation to the SMP can not be recognized under the light microscope, unlike ZIO preparations (Fig. 13).

However, under the electron microscope, cells with dense deposits due to reaction products of DAB and O_SO_4 are observed along the circular muscle-submucosa interface (Fig. 15). The immunoreaction products with vimentin-antiserum make the fine structure of the cells indistinct again, but the gap junction-rich cells are clearly distinguished by their characteristic gap junctions (Fig. 14) from typical fibroblasts that are likely to contain these immunoreaction products.

The nerve fibers and the smooth muscle cells were completely unstained.

Discussion

The present study clearly demonstrates a special type of interstitial cell that is characterized by many large gap junctions and is closely associated with the SMP and circular muscle layer of the guinea-pig proximal colon. Their ultrastructural features are similar to both fibroblasts (with well-developed Golgi apparatus and RER, many mitochondria and abundant intermediate filaments) and smooth muscle cells (with basal laminae and many caveolae), and seem to correspond to those of ICC of the canine colon (Berezin et al. 1988), though the presence of thick filaments is reported in the latter (Torihashi et al. 1994). Their superficial position is also consistent with ICC of the canine colon in which the gap junctions were found only within the first 1–3 cellular layers of the circular muscle layer close to the ICC reticulum (Berezin et al. 1988).

Recent electrophysiological studies in the canine colon appear to support the opinion that ICC at the circular muscle-submucosa interface are involved in the pacemaking activity (Berezin et al. 1988): spontaneous slow waves were recorded in the preparations of the circular muscle layers containing ICC (Smith et al. 1997; Serio et al. 1991; Liu et al. 1992, 1993). It was also reported that slow waves are selectively abolished in a correlation with selective damage of ICC at the circular muscle-submucosa interface of the canine colon by selective methylene-blue staining following by strong light illumination (Liu et al. 1994). Therefore, it is likely that the gap junction-rich cells in the present study also act as pacemakers in the guinea-pig proximal colon.

Meanwhile, the three types of interstitial cell found in the guinea-pig DMP (Zhou and Komuro 1992a, b) could not be distinguished in the present observation, in spite of the fact that the SMP of the colon is suggested as corresponding to the DMP of the small intestine (Rumessen and Thuneberg 1982). The cells of the SMP appear to resemble best the gap junction-rich cells of the DMP (Zhou and Komuro 1992a, b), though branching cytoplasmic processes have not been shown in the latter cells. Two others, glycogen-rich cells and the fibroblast-like cells with small gap junctions in the small intestine (Zhou and Komuro 1992a, b), were not observed in the colon.

It is an interesting question how these region differences in cell type correlate to the functional properties within the gastro-intestinal tract. They may represent different regulatory mechanisms required for different types of motility. In this context, it may be worth noting that the cellular connection by large gap junctions has not been shown in ICC of the human colon (Rumessen et al. 1993).

Reprocessing of whole-mount preparations for electron microscopy by the ZIO method and by vimentin-immunohistochemistry clearly revealed that the gap junction-rich cells associated with the SMP of the guinea-pig colon have a stellate form resembling ICC (Cajal 1893, 1911). This observation, together with the fact that the ZIO method provides a similar staining affinity to methylene blue (Taxi 1965; Thuneberg 1982), seems to indicate that this type of cell corresponds to ICC. Similar stellate cells to these are also observed in the canine colon by NADH-diaphorase histochemistry (Xue et al. 1993).

Since substantial amounts of vimentin filaments are detected in the present study, it is likely that this putative ICC of the guinea-pig colon is mesenchymal in origin. Immunoreactivity for vimentin in probable ICC has been reported in rat (Komuro 1987) guinea-pig (Tokui et al. 1992) small intestines and in the canine colon (Torihashi et al. 1994).

References

Berezin I, Huizinga JD, Daniel EE (1988) Interstitial cells of Cajal in the canine colon: a special communication network at the inner border of the circular muscle. J Comp Neurol 273:42–51

Berezin I, Huizinga JD, Daniel EE (1990) Structural characterization of interstitial cells of Cajal in the myenteric plexus and muscle layers of canine colon. Can J Physiol Pharmacol 68:1419–1431

Cajal SR (1893) Sur les ganglions et plexus nerveux de l'intestin. C R Soc Biol Paris 45:217–223

Cajal SR (1911) Histologie du système nerveux de l'homme et des vertébrés. Tome 2. Maloine, Paris

Christensen J (1992) A commentary on the morphological identification of interstitial cells of Cajal in the gut. J Auton Nerv Syst 37:75–88

Conklin JL, Du C (1990) Pathways of slow-wave propagation in proximal colon of cats. Am J Physiol 258:G894–G903

Du C, Conklin JL (1989) Origin of slow waves in the isolated proximal colon of the cat. J Auton Nerv Syst 28:167–178

Faussone-Pellegrini MS (1985) Ultrastructural peculiarities of the inner portion of the circular layer of the colon. II. Research on the mouse. Acta Anat 122:187–192

Faussone-Pellegrini MS, Cortesini C, Pantalone D (1990) Neuromuscular structures specific to the submucosal border of the human colonic circular muscle layer. Can J Physiol Pharmacol 68:1437–1446

Komuro T (1987) Three-dimensional structure of the intestinal wall. Ther Res 7:107–115

Liu LWC, Daniel EE, Huizinga JD (1992) Excitability of canine colon circular muscle disconnected from the network of interstitial cells of Cajal. Can J Physiol Phamacol 70:289–295

Liu LWC, Thuneberg L, Daniel EE, Huizinga JD (1993) Selective accumulation of methylene blue by interstitial cells of Cajal in canine colon. Am J Physiol 264:G64–G73

Liu LWC, Thuneberg L, Huizinga JD (1994) Selective lesioning of interstitial cells of Cajal by methylene blue and light leads to loss of slow waves. Am J Physiol 266:G485–G496

Rumessen JJ, Thuneberg L (1982) Plexus muscularis profundus and associated interstitial cells. I. Light microscopical studies of mouse small intestine. Anat Rec 203:115–127

Rumessen JJ, Thuneberg L, Mikkelsen HB (1982) Plexus muscularis profundus and associated interstitial cells. II. Ultrastructure studies of mouse small intestine. Anat Rec 203:129–146

Rumessen JJ, Peters S, Thuneberg L (1993) Light- and electron microscopical studies of interstitial cells of Cajal and muscle cells at the submucosal border of human colon. Lab Invest 68: 481–495

Serio R, Barajas-Lopez C, Daniel EE, Berezin I, Huizinga JD (1991) Slow-wave activity in colon: role of network of submucosal interstitial cells of Cajal. Am J Physiol 260:G636–G645

Smith TK, Reed JB, Sanders KM (1987) Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. Am J Physiol 252:C215–C224

Stach W (1972) Der Plexus entericus extremus des Dickdarmes und seine Beziehungen zu den interstitiellen Zellen (Cajal). Z Mikrosk Anat Forsch 85:245–272

Taxi J (1965) Contribution a l'étude des connexions des neurones moteurs due système nerveux autonome. Ann Sci Nat Zool Biol Anim 7:413–674

Thuneberg L (1982) Interstitial cells of Cajal: intestinal pacemaker cells? Adv Anat Embryol Cell Biol 71:1–130

Thuneberg L (1989) Interstitial cells of Cajal. In: Wood JD (ed) Handbook of physiology. The gastrointestinal system, vol 1. American Physiological Society, Bethesda, Md, pp 349–386

Tokui K, Zhou DS and Komuro T (1992) Immunohistochemical study of the interstitial cells of Cajal. J Smooth Muscle Res 28:380–382

Torihashi S, .Gerthoffer WT, Kobayashi S, Sanders KM (1994) Identification and classification of interstitial cells in the canine proximal colon by ultrastructure and immunocytochemistry. Histochemistry 101:169–183

Ward SM, Kellar RG, Sanders KM (1991) Structure and organization of the electrical activity of the canine distal colon. Am J Physiol 260:G724–G735

Xue C, Ward SM, Shuttleworth CW, Sanders KM (1993) Identification of interstitial cells in canine proximal colon using NADH diaphorase histochemistry. Histochemistry 99:373–384

Zhou DS, Komuro T (1992a) Interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine, with special reference to the interstitial cells of Cajal. Cell Tissue Res 268:205–216

Zhou DS, Komuro T (1992b) The cellular network of interstitial cells associated with the deep muscular plexus of the guinea pig small intestine. Anat Embryol 186:519–527

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Ultrastructural identification of the *c-kit*-expressing interstitial cells in the rat stomach: a comparison of control and Ws/Ws mutant rats

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Abstract. Interstitial cells in the circular muscle layer of the stomach of the Ws/Ws mutant rat, which lacks *c-kit*-expressing cells, and its siblings have been studied by electron microscopy. In the sibling control rats, two types of interstitial cells are found lying in close association with nerve bundles. Cells of the first type are characterized by electron-dense cytoplasm containing abundant mitochondria, granular endoplasmic reticulum, and Golgi apparatus. Intermediate filaments are richly distributed throughout the perinuclear region and the cell processes. Caveolae, subsurface cisterns, and indistinct basal lamina are observed along the cell membrane. The most conspicuous feature of this cell type is the existence of many large gap junctions that interconnect with the same type of cell, smooth muscle cells, or cells of the second type. Cells of the second type show an ultrastructure similar to fibroblasts, viz., a well-developed Golgi apparatus and granular endoplasmic reticulum whose cisterns often show a dilated form and contain flocculent material. Unlike typical fibroblasts, however, cells of this type also form many gap junctions with cells of the first type and smooth muscle cells. Both types of cells are observed in close apposition to nerve varicosities. Since cells of the first type are absent in the Ws/Ws mutant rat, we concluded that they correspond to *c-kit*-expressing cells and to interstitial cells of Cajal.

Key words: Interstitial cells of Cajal (ICC) – Digestive organ – Pacemaker – Morphology – Rat (Ws/Ws)

Introduction

Postnatal blockade of the cells that express *c-kit* tyrosine kinase receptor by injection of an anti-*c-kit* receptor monoclonal antibody (ACK2) results in severe abnormality of intestinal motility in the mouse small intestine (Maeda et al. 1992) and colon (Torihashi et. al. 1995). Huizinga et al. (1995) have shown, by whole-mount RNA in situ experiments, that interstitial cells of Cajal (ICC) in the small intestine of the mouse express *c-kit* messenger RNA. Immunohistochemical studies with ACK2 also indicate that the ACK2-positive cells in the guinea-pig small intestine show almost identical shapes and distribution patterns to those of ICC (Komuro and Zhou 1996; Komuro et al. 1996).

The *c*-kit receptor is encoded by the *W* locus in the mouse (Chabot et al. 1988; Geissler et al. 1988) and the *Ws* locus in the rat (Tsujimura et al. 1991). Ward et al. 1994, 1995) have found a remarkable reduction of ICC in the myenteric plexus region of the small intestine in the W/W^v mutant mouse and in the Sl/Sl^d mutant mouse, which has mutations in the steel cell factor, the ligand for the *c-kit* receptor. Furthermore, Isozaki et al. (1995) have demonstrated that the *c-kit* expressing cells detected by in situ hybridization are not found in the external muscle layer of the stomach of Ws/Ws mutant rats, which show spontaneous mutation at the *Ws* locus.

These studies strongly suggest that the *c-kit* receptor plays a crucial role in the development of normal gastrointestinal motility and that these *c-kit*-expressing cells correspond to ICC, which have been proposed as the pacemakers of intestinal peristalsis (Thuneberg 1982). However, the ultrastructure of *c-kit* expressing cells and their equivalence to ICC remains controversial. Ws/Ws rats should be useful for examining whether the absence of *c-kit*-expressing cells results in the absence of morphologically identifiable ICC. The aim of the present study has been to identify the interstitial cells closely associated with the nerve bundles in the circular muscle layer of the stomach in Ws/Ws mutant rats and their normal siblings, and to characterize the

This paper is dedicated to Professor Andreas Oksche on the occasion of his 70th birthday, in recognition of out standing contributions to neuroscience and to the internationalisation of science

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ultrastructural features of *c-kit*-expressing cells that correspond to ICC.

Materials and methods

Electron microscopy

Homozygous Ws/Ws mutant rats and sibling control +/+ rats were used. Animals were anesthetized by ether inhalation and the stomach was moderately inflated with phosphate-buffered saline (PBS). Following perfusion through the left ventricle of the heart with a fixative containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4° C, the corpus and the antrum were removed, cut into small pieces and placed in the same fixative for 3 h. The specimens were then rinsed in the same buffer, post-fixed in 1% osmium tetroxide in the same buffer for 2 h at 4° C, rinsed in distilled water, block-stained in a saturated solution of aqueous uranyl acetate for 2 h, dehydrated in a graded series of ethyl alcohols, and embedded in Epon epoxy resin. Ultrathin sections were cut on a Reichert ultramicrotome and double-stained with uranyl acetate and lead tartrate for observation under a JEOL JEM 1200EX electron microscope.

In situ hybridization

Under terminal anesthesia with ether, the stomach was removed from each animal, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, overnight, and embedded in paraffin. Serial sections were cut at a thickness of 3 μm. Details of the in situ hybridization technique have been described previously (Hirota et al. 1992). Digoxigenin(DIG)-labeled single-strand RNA probes were prepared by using a DIG RNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany), according to the manufacturer's instructions. Hybridization of *c-kit* mRNAs was performed at 50° C for 16 h, and the signals were detected by using a nucleic acid detection kit (Boehringer Mannheim Biochemica). The controls included hybridization with the sense probes, ribonuclease treatment before hybridization, and the use of neither the antisense RNA probe nor the anti-digoxigenin antibody. To exclude the possibility that the *c-kit* mRNA-expressing cells were mast cells, the adjacent section was stained with Alcian blue and Nuclear fast red.

Results

Control +/+ rat

The circular muscle layers of the antrum and corpus of the control +/+ rat are composed of about 60 and 30 layers of smooth muscle cells, respectively, and are divided by connective tissue septa containing nerve bundles and blood vessels (Fig. 1). Distinct nerve plexuses, such as the deep muscular plexus (DMP) in the small intestine or submuscular plexus (SMP) in the colon, have not been identified in the rat stomach. However, nerve bundles are scattered throughout the circular muscle layer and run mainly parallel to the axis of the circular muscle cells. A relatively small number of nerves are found within the innermost 10 cell layers. Some free cells, including eosinophils and macrophages, are occasionally observed in the interstitium around these nerve bundles.

Apart from Schwann cells, two other cell types are found in close association with the nerve bundles. Cells of the first type have a longer cellular axis parallel to the circular muscle cells (Fig. 2) and project cytoplasmic processes extending in various directions (Figs. 2, 3). The nuclei are oval and heterochromatin is distributed at the periphery. The most striking feature of this type of cell is the presence of many large gap junctions interconnecting with each other (Fig. 3), with neighboring smooth muscle cells (Fig. 2), and with cells of the second type (see Fig. 6) described below. Their cytoplasm usually shows a higher electron-density than that of adjacent muscle cells and cells of the second type. They contain abundant mitochondria and well-developed Golgi apparatus and rough endoplasmic reticulum (RER) in perinuclear regions (Figs. 2, 3). The basal lamina is indistinct, but caveolae are often observed along the cell membranes (Fig. 2). Bundles of intermediate filaments are seen throughout the cytoplasm (Fig. 4). Cilia or basal bodies are occasionally encountered. Lipid droplets are also seen. Cells of the first type are rarely found within the 5~10 muscle cell layers from the circular muscle-submucosa interface.

Cells of the second type are also elongated and have the same axis as those of the circular muscle cells (Fig. 5). The nuclei of these cells are frequently oval with smooth contours. Their cytoplasm is usually less electron-dense than that of adjacent muscle cells or the first type of cell (Figs. 5, 6). They show cytoplasmic features similar to those of the fibroblasts and contain well-developed Golgi apparatus and RER of which the cisterns are often dilated and contain flocculent substances. There is no basal lamina. Cilia or basal bodies and lipid droplets are occasionally seen. Although their ultrastructure resembles that of fibroblasts, they often form gap junctions with cells of the first type and adjacent smooth muscle cells. However, these gap junctions are usually smaller in size and less frequent than those of the first cell type. Gap junctions between cells of the second type have not been found.

Nerve varicosities containing many synaptic vesicles are often observed in close association with both the first and the second cell types (Figs. 2, 3), although the latter case is not illustrated. These nerve varicosities are dominated by one of three types of synaptic vesicles: small clear round vesicles, small clear flattened vesicles, or large cored vesicles.

Cells of the second type are occasionally found at the circular muscle-submucosa interface. They extend their processes into the connective tissue septum to form gap junctions with the muscle cells within the circular muscle layer (Figs. 7, 8).

Ws/Ws mutant rat

The basic structure of the circular muscle layer of the stomach of the Ws/Ws mutant rat shows no difference from that of controls. The distribution and number of nerve bundles and blood vessels seems normal. However, the cells of the first type are not found in the antrum and

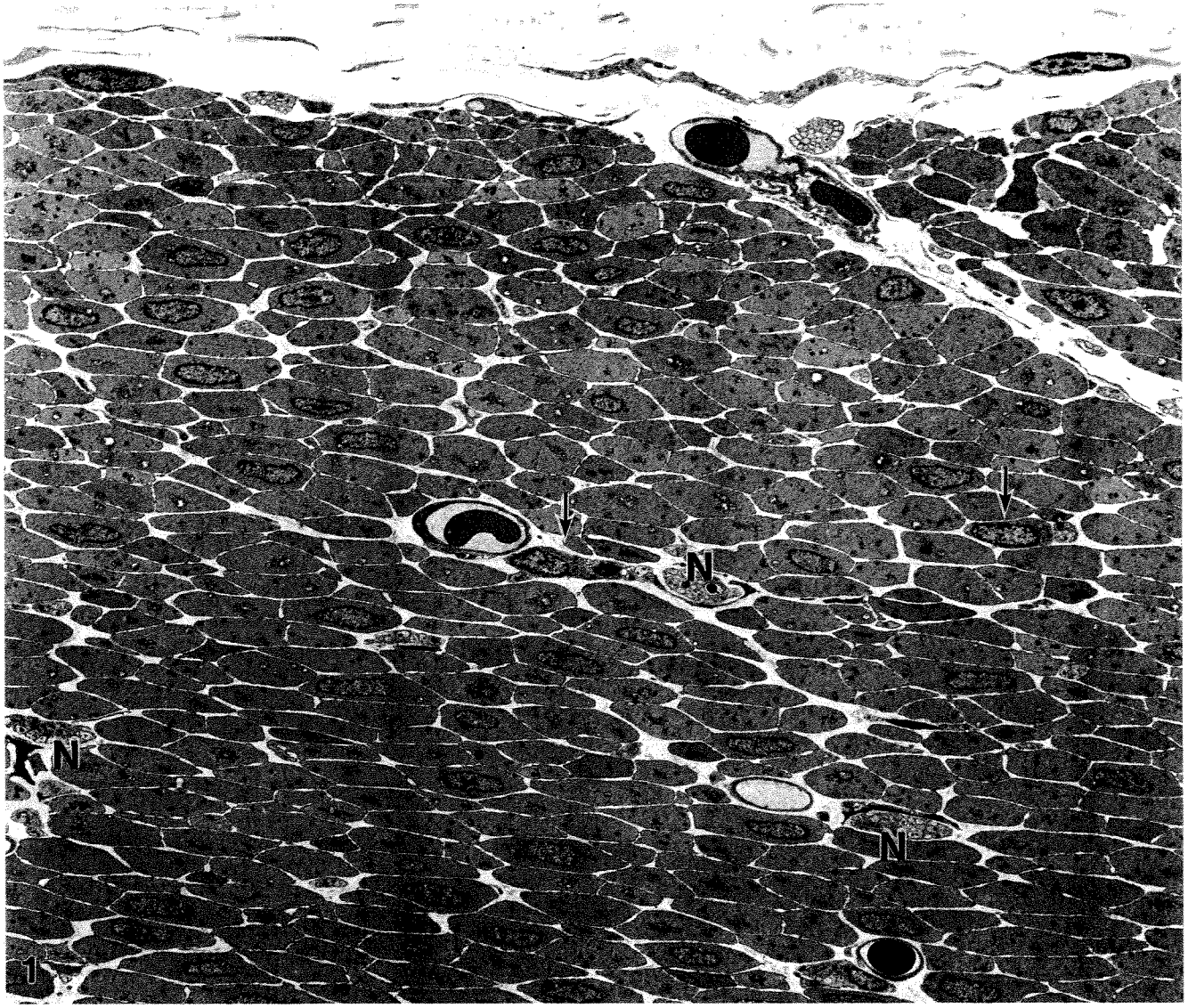
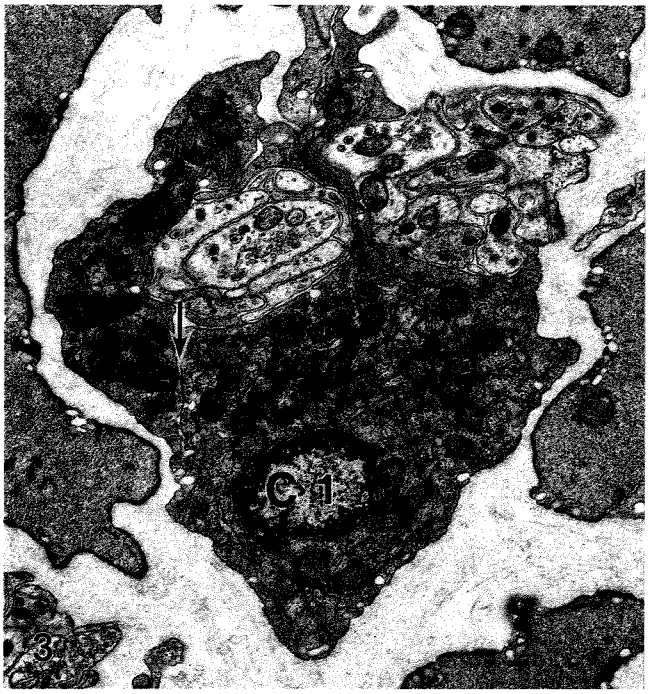


Fig. 1. Electron micrograph showing a cross-section of the circular muscle layer of the control rat antrum. Nerve bundles (*N*) have no specific distribution but are dispersed within the muscle layer together with blood capillaries. Interstitial cells (*arrows*) are found in close apposition to these nerve bundles. ×1500

Fig. 2. First type of gap-junction-rich interstitial cell (*C1*) in the control antrum. It makes gap junctions (*arrow*) with the adjoining smooth muscle cell. The cytoplasm shows high electron-density. Mitochondria, RER, and caveolae (*arrowheads*) can be seen. A nerve terminal (*N*) is observed in close contact with this type of cell. ×18000. *Inset*: Higher magnification of the gap junction, indicated by the *arrow*, between C1 and the smooth muscle cell. ×60000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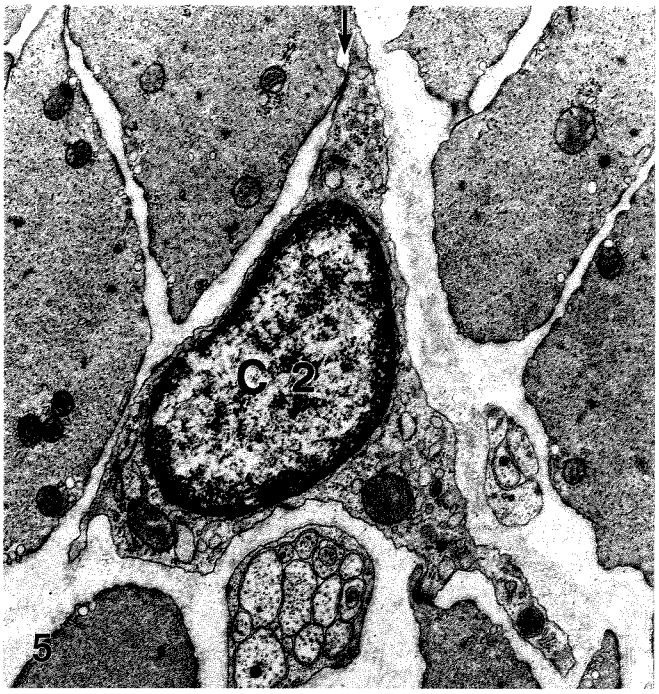
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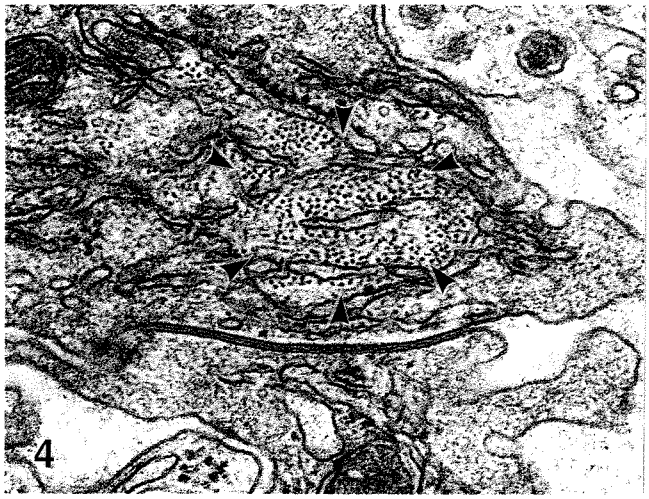
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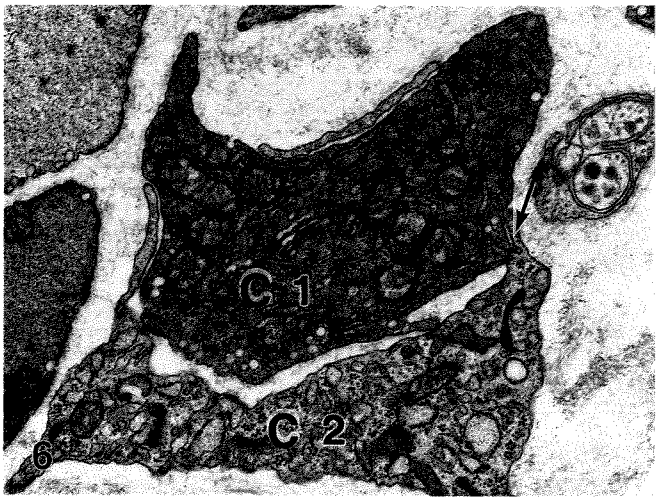
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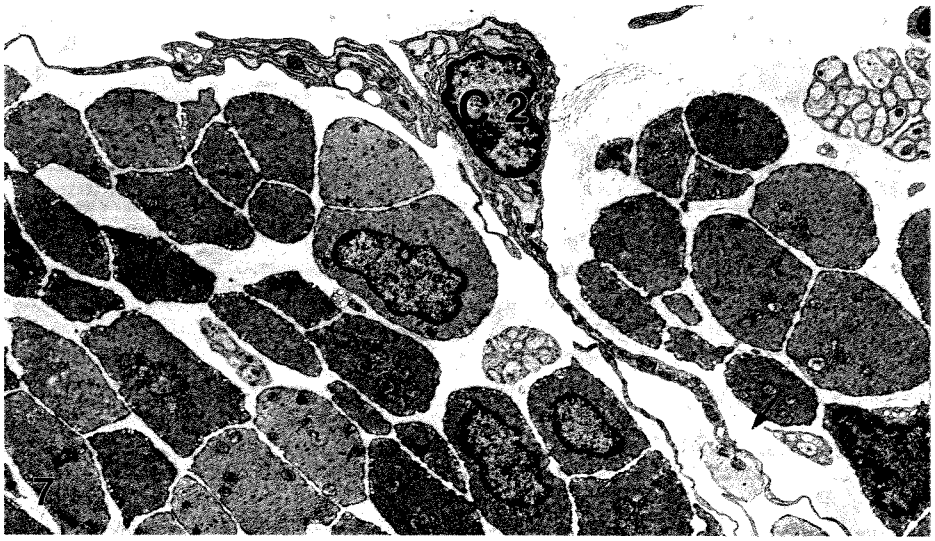
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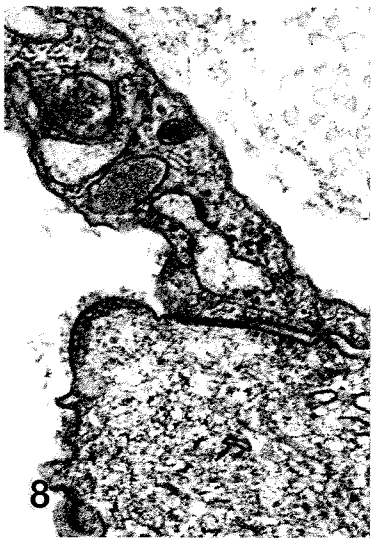
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corpus of the mutant rat stomach. Neither the cell body not the small cytoplasmic processes identified as belonging to the first type of cell have been observed so far.

Unlike those of the first cell type, cells of the second type are regularly observed in both the antrum (Figs. 9, 10) and corpus and seem to retain a normal distribution, although quantitative data have not been evaluated. As in control animals, they show cytoplasmic features similar to those of fibroblasts, but with a low electron-density of the cytoplasm. They form gap junctions with the circular smooth muscle cells. As serial sections indicate (Figs. 9, 10), they are characterized by a well-developed RER whose cisterns are often filled with flocculent material. An accumulation of abundant mitochondria has never been observed in any of the profiles of the second type of cell.

In situ hybridization

The expression of *c-kit* was examined by in situ hybridization in the stomach of +/+ control rats and Ws/Ws mutant rats. In the control rats, cells expressing *c-kit* mRNA were located in the circular muscle layer and the myenteric plexus region of the corpus (Fig. 11). In contrast to the control rats, no cells expressing *c-kit* mRNA were detectable in the circular muscle layer or the myenteric plexus region of the corpus of the mutant rats (Fig. 12). The cells expressing *c-kit* mRNA in the circular muscle layer of control rats were considered not to represent mast cells, since most Alcian-blue-positive mast cells were located in the submucosal layer and very few were observed in the muscle layer.

Discussion

The present study has revealed that the first type of interstitial cell is absent from the stomach of the Ws/Ws rat, whereas the second type can be observed with a similar frequency to that of the control rats. Since cells expressing *c-kit* mRNA are also not detectable by in situ hybridization in the Ws/Ws rat stomach, the first type of cell probably corresponds to the cells expressing *c-kit* mRNA in the rat stomach. Moreover, this first type of cell is probably responsible for the regulation of gastric contraction, because a deficiency of cells expressing *c-kit* mRNA causes an abnormality in the pyloric sphincter function resulting in a significant reflux of bile to the stomach in Ws/Ws rats (Isozaki et al. 1995). Therefore, it can be concluded that the first cell type, which is characterized by large gap junctions, abundant mitochondria, and a highly electron-dense cytoplasm conforms to ICC in the rat stomach, as previously suggested by evidence that ICC in the mouse small intestine express *c-kit* mRNA (Huizinga et al. 1995) and that the myenteric ICC of the guinea-pig small intestine show immunoreactivity to anti-*c-kit* receptors (Komuro and Zhou 1996). This first type of interstitial cell in the rat stomach appears to correspond to ICC of the dog stomach, which are described as having an electron-dense cytoplasm (Daniel et al. 1984, 1989), and also to ICC of the human stomach, which are characterized by abundant intermediate filaments (Faussone-Pellegrini et al. 1989).

On the other hand, the second type of cell in the present study probably corresponds to fibroblast-like ICC (Daniel et al. 1989) and shows close similarities to fibroblast-like cells in the DMP region of the guinea-pig small intestine (Zhou and Komuro 1992a, b) and the rat small intestine (Komuro and Seki 1995), although their gap junctions seem to be larger in size and number than those of the small intestine.

Considering the multi-function of the family of fibroblast-like cells (Komuro 1990), the second type of cell probably constitutes a different mode of cell-to-cell communication system from the first type of interstitial cell or ICC. Further, interstitial cells forming many gap junctions are distributed normally in the DMP region of the Sl/Sl^{d} mutant mouse (Ward et. al 1995) and Ws/Ws mutant rat (Horiguchi et al. 1995). These observations suggest that the DMP region of these species contain gap-junction-forming cells of which development does not depend on *c-kit* receptors.

Regarding interstitial cells forming gap junctions, only cells of one type have been reported in the SMP of the colon in the species studied so far, i.e., dog (Berezin et al. 1988; Liu et al. 1993), human (Rumessen et al. 1993), and guinea-pig (Ishikawa and Komuro 1996). However, in the DMP region of the circular muscle layer of the small intestine, cells of one type have been reported in mouse (Rumessen et al. 1982; Thuneberg 1982), human (Rumessen et al. 1992), and dog (Torihashi et al. 1993), whereas two types, i.e., gap-junction-rich cells and fibroblast-like cells, are present in the rat (Komuro and Seki 1995), and three types, i.e., gap-junction-rich cells, glycogen-rich cells, and fibroblast-like cells are found in guinea-pig (Zhou and Komuro 1992a, b). An interesting subject for future studies is whether these differences truly represent features peculiar to the tissues or species,

Fig. 3. Cross-section of the first type of cell (*C1*) in the control rat corpus. Abundant mitochondria are crowded in the perinuclear cytoplasm. *Arrow*, Gap junction between cells of the same type. ×13500

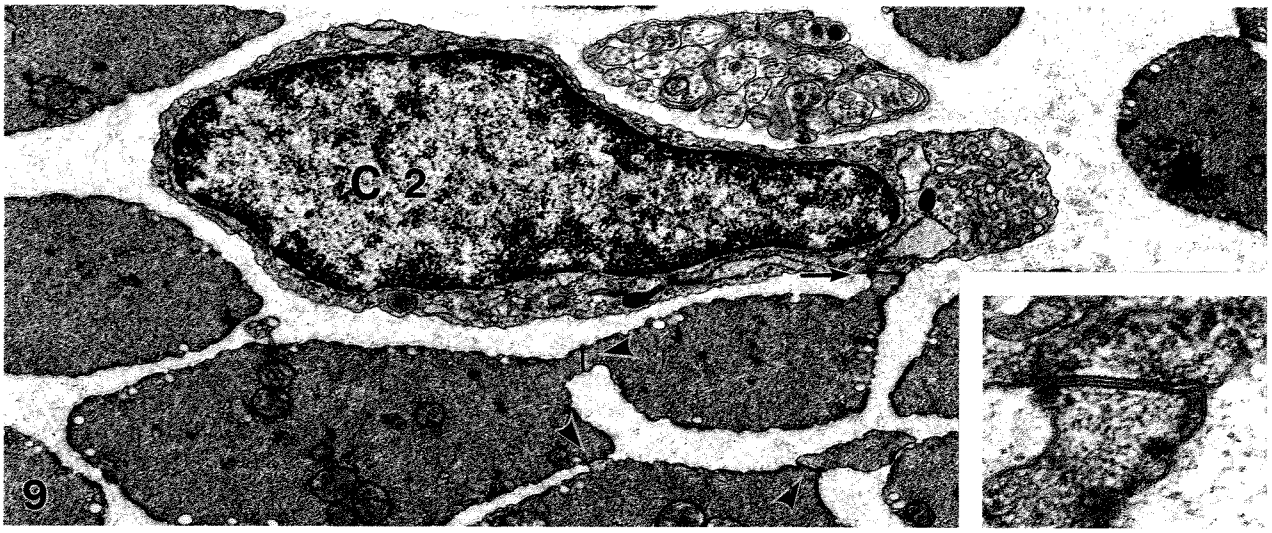
Fig. 4. Gap junction between the first type of cells. Intermediate filaments are richly distributed in the cytoplasm (*arrowheads*). ×50000

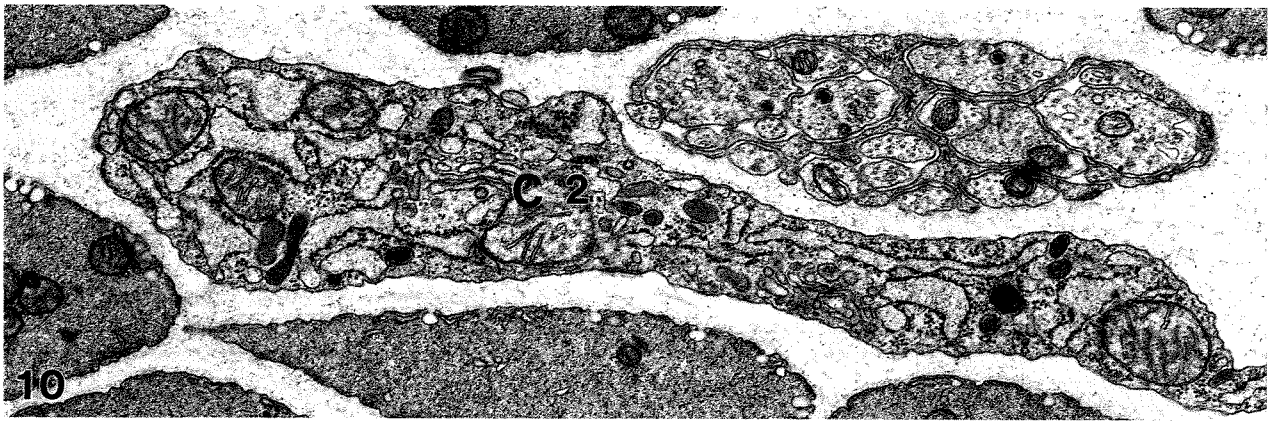
Fig. 5. Second type of gap-junction-rich interstitial cell (*C2*) in the control rat antrum. The cytoplasm is less electron-dense and contains dilated cisterns of RER. *Arrow*, Gap junction between the second type of cell and a smooth muscle cell. ×15500

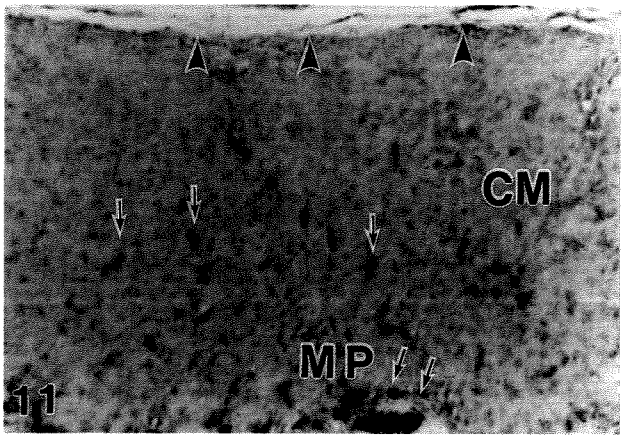
Fig. 6. Gap junction (*arrow*) between the first (*C1*) and second type of cells (*C2*). Note the difference in the cell organelles and cytoplasmic electron-density in the two cell processes. ×17000

Fig. 7. Second type of cell (*C2*) at the circular muscle-submucosa interface of the antrum forming a gap junction with the muscle cell (*arrow*). ×5000

Fig. 8. Higher magnification of the cytoplasmic process indicated by the arrow in Fig. 7, showing a gap junction between the second type of cell and smooth muscle cell. ×50000







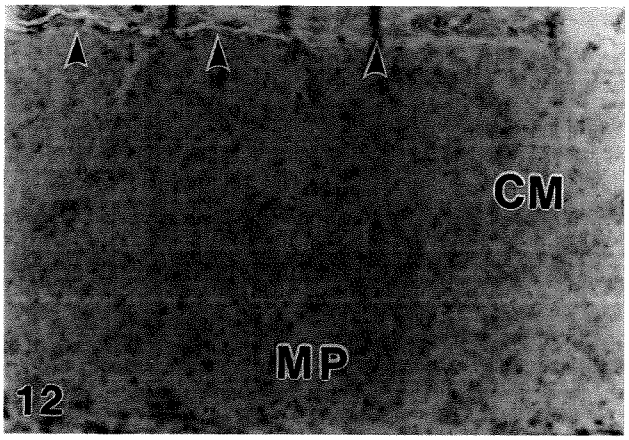


Fig. 9. The second type of cell (*C2*) in the circular muscle layer of the antrum of the Ws/Ws mutant rat. Well-developed Golgi apparatus and a few cisterns of RER can be seen in the cytoplasm, which has low electron-density. Gap junctions between the second type of cell and a smooth muscle cell (*arrow*), and between smooth muscle cells (*arrowheads*) can be observed. ×16000. *Inset*: Higher magnification of the gap junction between the second type of cell and the smooth muscle cell indicated by the *arrow*. ×80000

Fig. 10. A neighboring section of the same cell (*C2*) as that in Fig. 9, showing well-developed RER containing flocculent material in dilated cisterns. ×20000

Fig. 11. In situ hybridization of *c-kit* mRNA in the control rat corpus. The *c-kit*-expressing cells (*arrows*) are distributed in the myenteric plexus region (*MP*) and in the circular muscle layer (*CM*), excluding about one third of the layer from the circular muscle-submucosa interface (*arrowheads*). ×150

Fig. 12. In situ hybridization of *c-kit* mRNA in the mutant rat corpus. No *c-kit*-expressing cells are observed in the myenteric plexus region (*MP*) or in the circular muscle layer (*CM*). *Arrowheads*, Circular muscle-submucosa interface. ×150

and which type of cell has the most essential function in the regulatory mechanism of peristaltic movement.

References

Berezin I, Huizinga JD, Daniel EE (1988) Interstitial cells of Cajal in the canine colon: a special communication network at the inner border of the circular muscle. J Comp Neurol 273: 42–51

Chabot B, Stephenson DA, Chapman VM, Besmer P, Bernstein A (1988) The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. Nature 335:88-89

Daniel EE, Sakai Y, Fox JET, Posey-Daniel V (1984) Structural basis for function of circular muscle of canine corpus. Can J Physiol Pharmacol 62:1304–1314

Daniel EE, Berezin I, Allescher HD, Manaka H, Posey-Daniel V (1989) Morphology of the canine pyloric sphincter in relation to function. Can J Physiol Pharmacol 67:1560–1573

Faussone-Pellegrini MS, Pantalone D, Cortesini C (1989) An ultrastructural study of the interstitial cells of Cajal of the human stomach. J Submicrosc Cytol Pathol 21:439–460

Geissler EN, Ryan MA, Houseman DE (1988) The dominant white spotting (*W*) locus of the mouse encodes the *c-kit* proto-oncogene. Cell 55:185–192

Hirota S, Ito A, Morii E, Wanaka A, Tohyama M, Kitamura Y, Nomura S (1992) Localization of mRNA for *c-kit* receptor and its ligand in the brain of adult rats: an analysis using in situ hybridization histochemistry. Mol Brain Res 15:47-54

Horiguchi K, Zhou DS, Seki K, Komuro T, Hirota S, Kitamura Y (1995) Morphological analysis of *c-kit* expressing cells, with reference to interstitial cells of Cajal. J Smooth Muscle Res 31:303–305

Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A (1995) *W/kit* gene required for intestinal pacemaker activity. Nature 373:347–349

Ishikawa K, Komuro T (1996) Characterization of the interstitial cells associated with the submuscular plexus of the guinea-pig colon. Anat Embryol 194:49–55

Isozaki K, Hirota S, Nakama A, Miyagawa J, Shinomura Y, Xu ZD, Nomura S, Kitamura Y (1995) Disturbed intestinal movement, bile reflex to the stomach, and deficiency of *c-kit*-expressing cells in Ws/Ws mutant rats. Gastroenterology 109:456–464

Komuro T (1990) Re-evaluation of fibroblasts and fibroblast-like cells. Anat Embryol 182:103–112

Komuro T, Seki K (1995) Fine structural study of interstitial cells associated with deep muscular plexus of the rat small intestine, with special reference to the intestinal pacemaker cells. Cell Tissue Res 282:129–134

Komuro T, Zhou DS (1996) Anti-*c-kit* protein immunoreactive cells corresponding to the interstitial cells of Cajal in the guinea-pig small intestine. J Auton Nerv Syst 61:169–174

Komuro T, Tokiu K, Zhou DS (1996) Identification of the interstitial cells of Cajal. Histol Histopathol 11:769–786

Liu LWC, Thuneberg L, Daniel EE, Huizinga JD (1993) Selective accumulation of methylene blue by interstitial cells of Cajal in canine colon. Am J Physiol 264:G64–G73

Maeda H, Yamagata A, Nishikawa S, Yoshinaga K, Kobayashi S, Nishi K, Nishikawa S-I (1992) Requirement of *c-kit* for development of intestinal pacemaker system. Development 116: 369–375

Rumessen JJ, Thuneberg L, Mikkelsen HB (1982) Plexus muscularis profundus and associated interstitial cells. II. Ultrastructure studies of mouse small intestine. Anat Rec 203:129–146

Rumessen JJ, Mikelsen HB, Thuneberg L (1992) Ultrastructure of interstitial cells of Cajal associated with deep muscular plexus of human small intestine. Gastroenterology 102:56–88

Rumessen JJ, Peters S, Thuneberg L (1993) Light- and electron microscopical studies of interstitial cells of Cajal and muscle cells at the submucosal border of human colon. Lab Invest 68: 481–495

Thuneberg L (1982) Interstitial cells of Cajal: intestinal pacemaker cells? Adv Anat Embryol Cell Biol 71:1–130

Torihashi S, Kobayashi S, Gerthoffer WT, Sanders KM (1993) Interstitial cells in deep muscular plexus of canine small intestine may be specialized smooth muscle cells. Am J Physiol 265:G638–G645

Torihashi S, Ward SM, Nishikawa S-I, Nishi K, Kobayashi S, Sanders KM (1995) *c-kit*-Dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res 280:97–111

Tsujimura T, Hirota S, Nomura S, Niwa Y, Yamazaki M, Tono T, Morii E, Kim HM, Kondo K, Nishimune Y, Kitamura Y (1991) Characterization of Ws mutant allele of rats: a 12-base deletion in tyrosine kinase domain of *c-kit* gene. Blood 78:1942–1946

Ward SM, Burns AJ, Torihashi S, Sanders KM (1994) Mutation of the proto-oncogene *c-kit* blocks development of interstitial cells and electrical rhythmicity in murine intestine. J Physiol (Lond) 480:91–97

Ward SM, Burns AJ, Torihashi S, Harney SC, Sanders KM (1995) Impaired development of interstitial cells and intestinal electrical rhythmicity in *steel* mutants. Am J Physiol 269: C1577–C1585

Zhou DS, Komuro T (1992a) Interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine, with special reference to the interstitial cells of Cajal. Cell Tissue Res 268:205–216

Zhou DS, Komuro T (1992b) The cellular network of interstitial cells associated with the deep muscular plexus of the guinea pig small intestine. Anat Embryol 186:519–527

Anat Embryol (1998) 197:135–141
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ORIGINAL ARTICLE

Keisuke Seki · Terumasa Komuro

Further observations on the gap-junction-rich cells in the deep muscular plexus of the rat small intestine

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Abstract Interstitial cells forming many large gap junctions in the region of the deep muscular plexus of the rat small intestine were studied by electron microscopy and by three-dimensional cell models reconstructed from serial ultrathin sections. Two different profiles of cells were observed. Cells of the first profile are characterized by an elongated cell shape and by less electron-dense cytoplasm, containing many mitochondria, well-developed Golgi apparatus and free ribosomes. They mainly connect with smooth muscle cells of the main circular layer. In a three-dimensional cell model, the total area of the gap junctions occupies 1.3% of the cell surface. Cells of the second profile are characterized by the frequent occurrence of slender cytoplasmic processes, higher electron-dense cytoplasm, containing mitochondria, Golgie apparatus and well-developed rough endoplasmic reticulum, and numerous caveolae on the cell membrane. In this cell model, gap junctions occupy 0.8% of the cell surface. The ratio of gap junctions with the same profile of cells to the total gap junction area is 37.7%, which is more than three times greater than the 9.9% in cells of the first profile. These cells were closely associated with nerve terminals. It is likely that these cells with different profiles constitute subtypes with each other and cooperate for regulation of intestinal motility via the transmission of nerve signals.

Key words Interstitial cells of Cajal · Ultrastructure · Gap junction · Intestine · Motility

Introduction

Cells regarded as interstitial cells of Cajal (ICC; Cajal 1911) have been reported in the deep muscular plexus (DMP) of the small intestine in various animals, including mouse (Rumessen and Thuneberg 1982; Rumessen et al. 1982), guinea-pig (Zhou and Komuro 1992a, b), dog (Torihashi et al. 1993), and rat (Komuro and Seki 1995), since their regulatory function for intestinal motility has been suggested (Thuneberg 1982). These cells are characterized by many large gap junctions that are believed to be an important feature of the regulatory cells. However, it is not certain whether the gap-junction-forming cells in these studies correspond to each other and to ICC in the original description by Cajal (Cajal 1911). In the guinea-pig DMP (Zhou and Komuro 1992a, b), for example, there are two types of interstitial cells forming many large gap junctions, i.e., the gap junction-rich cells and the glycogen-rich cells, in addition to the fibroblast-like cells forming a few small gap junctions. They all show some differences from the ICC-III in the mouse, which is the only type of gap junction-forming cell in that animal (Rumessen et al. 1982).

On the other hand, in a previous study on the rat DMP (Komuro and Seki 1995) we noticed some ultrastructural variations among the cells forming large gap junctions with respect to the electron density of the cytoplasm and to the distribution patterns of the cell organelles.

The present study aims to further elucidate the gap junction-rich cells associated with the rat DMP by detailed examination of serial ultrathin sections. Three-dimensional images of these cells were reconstructed from serial sections with aid of a computer graphic system.

Materials and methods

Transmission electron microscopy

Pieces of proximal jejunum from adult Wistar rats were fixed in Karnovsky's fixative containing 3% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.3, for 2 h at 4° C. The specimens were rinsed in the same buffer and post fixed in 1% osmium tetroxide for 2 h at 4° C. Following osmication, the specimens were rinsed in distilled water, block-stained with saturated uranyl acetate solution for 3 h, dehydrated in a graded series of ethyl alcohol and embedded in Epon epoxy resin. Ultrathin sections were cut using a Reichert microtome and were double-

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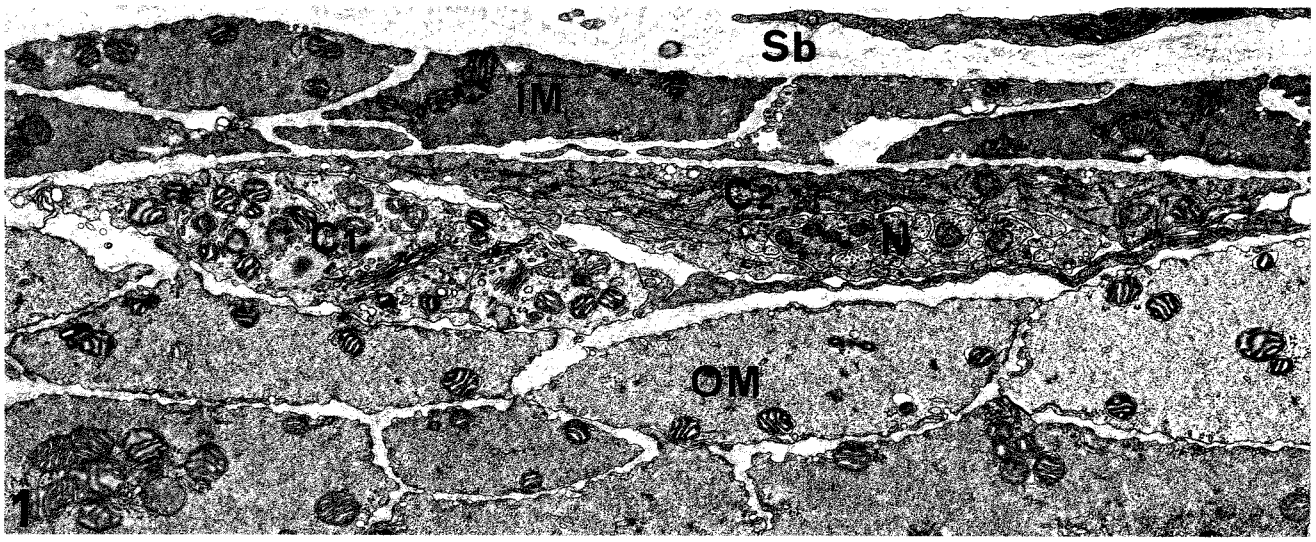
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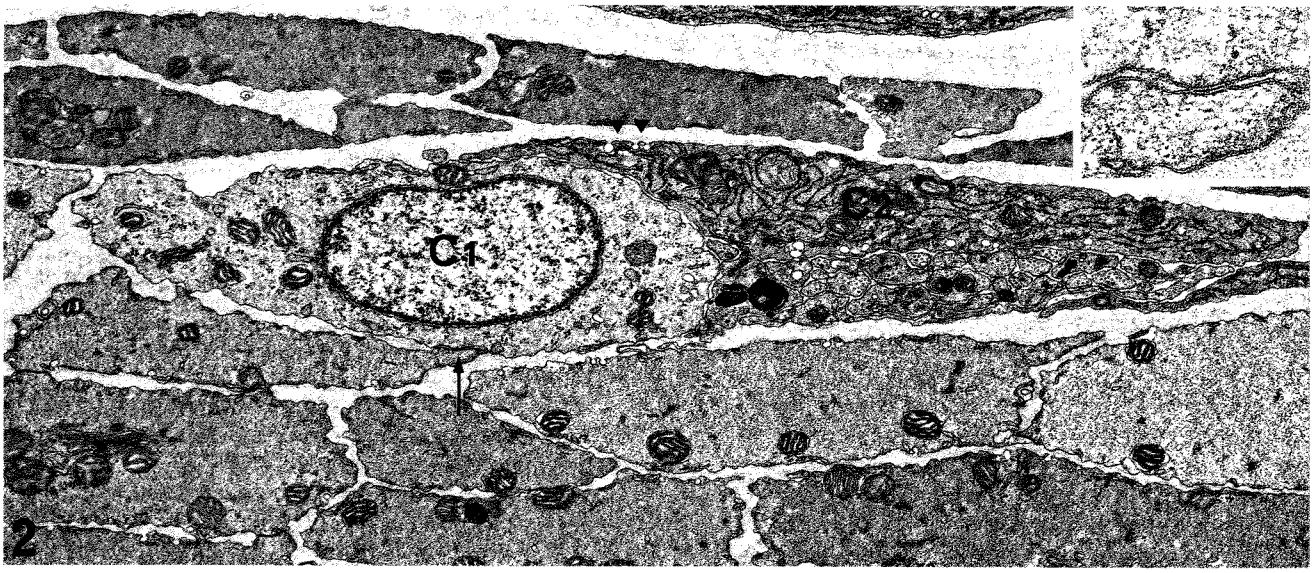
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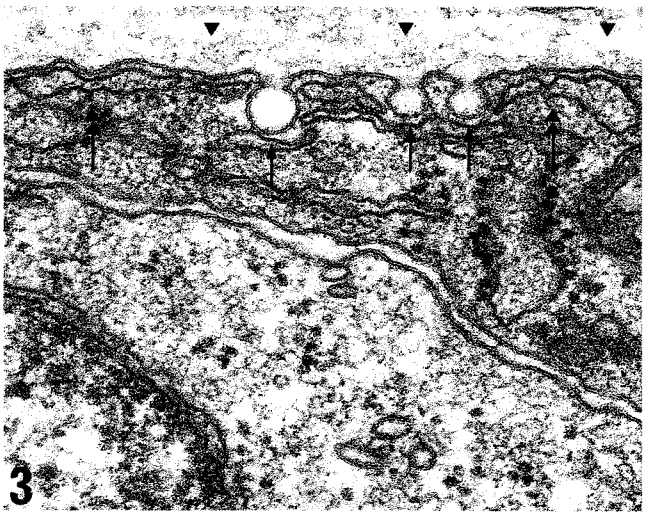
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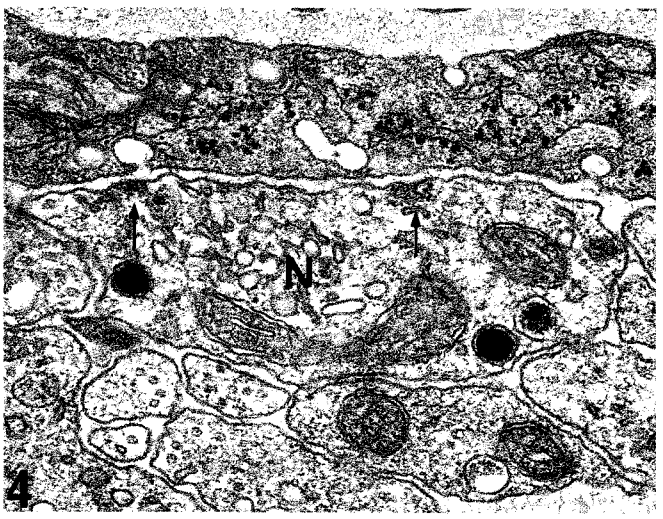
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Table 1 Measurements based on the reconstructed cell models (*GJ* gap junction)

Cell type	Cell surface (A) (μm^2)	Number of GJ	Total area (B) of GJ (μm^2)	GJ area (C) between GJ cells (μm^2)	B/A (%)	C/B (%)
C1	549.6	33	7.1	0.7	1.3	9.9
C2	1333.1	39	10.6	4.0	0.8	37.7

stained with uranyl acetate and lead citrate for observation under a JEM 1200 EXII electron microscope. In a preliminary examination of several sets (complete and half complete) of serial ultrathin sections, two distinct profiles of gap-junction-rich cells were distinguished, not only from single sections but from series of profiles. Thus, two sets of serial ultrathin sections (70 nm thick) containing identified target cells, placed on grids with slit meshes having eight sections to each grid, were selected for three-dimensional reconstruction models.

Reconstruction of cell models

Profiles of the target cells were photographed, using every fourth section. If necessary, profiles containing the images of each gap junction were also photographed in order to exactly reproduce the size of the gap junctions in the cell models. The photographs were then enlarged to a final magnification of ×20,000. Relevant structures of the cells were traced onto transparent plastic films. These features included the contours of the target cells, their nuclei, gap junctions, and adjacent smooth muscle cells of both inner and outer circular muscle layers. The cells were reconstructed with a computer graphic system (TRI, Ratoc System Engineering, Tokyo, Japan), and all parameters were calculated on the basis of these models.

Results

The DMP of the rat small intestine is located between the inner thin (1–3 cells thick) and outer main layers of the circular muscle (Fig. 1). The plexus consists of nerve bundles running parallel to the circular muscle cells and transverse interconnecting strands. These nerve bundles are closely associated with interstitial cells of different appearance that form many large gap junctions (Figs. 1, 2).

Fig. 1 Two different cell profiles closely associated with the nerve bundle of the DMP (*N*), which is located between the outer (*OM*) and the inner (*IM*) circular muscle layers beneath the submucosa (*Sb*). One (*C1*) is characterized by its less electron-dense cytoplasm and many mitochondria, while the other (*C2*) by its electron-dense cytoplasm and well-developed RER. ×14,000

Fig. 2 An electron micrograph of a neighboring section to that of Fig. 1. Both cell profiles show morphological consistency with those in Fig. 1, though the peri-nuclear cytoplasm of C1 shows a few mitochondria. A gap junction and caveolae are indicated by an *arrow* and *arrowheads*, respectively. ×15,000. **Inset** Higher magnification of the gap junction indicated by the *arrow*. ×60,000

Fig. 3 Higher magnification of the area indicated by the arrowheads in Fig. 2. Caveolae and subsurface cisterns are indicated by *arrows* and *double-headed arrows*, respectively. A basal lamina is also seen (*arrowheads*). ×90,000

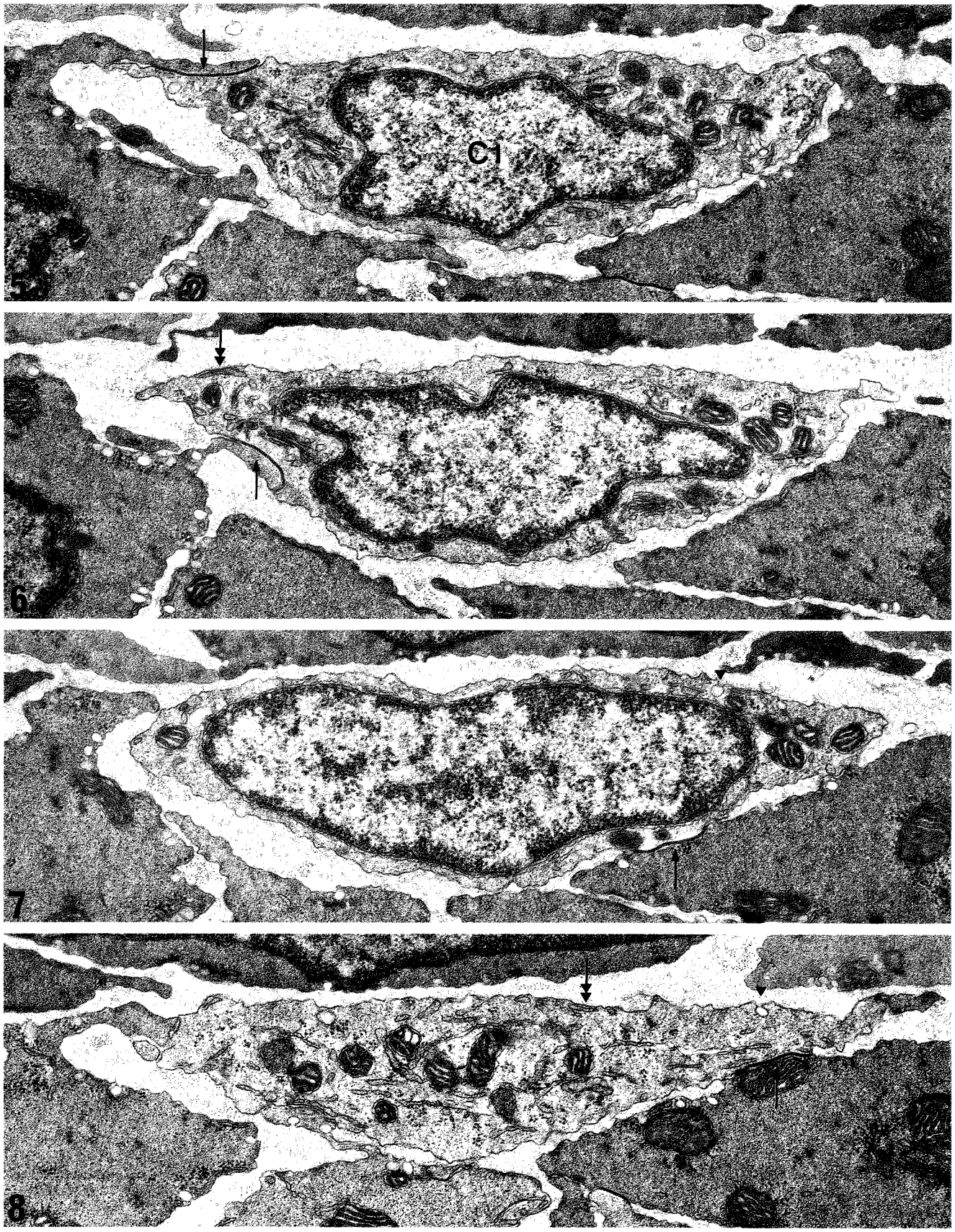
Fig. 4 Nerve varicosity (*N*) containing both small clear (about 50 nm) and large-cored (about 100 nm) vesicles closely associated with the second profile of cell. Patches of electron-dense materials are seen at the inner aspect of the axonal membrane (*arrows*). ×48,000

These gap-junction-rich cells share common cytoplasmic features, i.e., the presence of many cell organelles, including mitochondria, Golgi apparatus, smooth (SER) and rough (RER) endoplasmic reticulum, and cytoskeletal components such as microtubles, thin filaments and intermediate filaments. However, myosin filaments and dense bodies were not observed. Thus, their profiles are seemingly similar to the fibroblast-like cells (Komuro and Seki 1995), but they are distinguished from the fibroblast-like cells by the presence of the basal lamina and caveolae (Figs. 2, 3). They show close contacts with axon terminals containing synaptic vesicles (Fig. 4). In addition to these common features, gap-junction-rich cells show two different profiles, described below. They are usually distinguished by a simple difference of cytoplasmic electron density. Moreover their different cytoplasmic features are consistently observed in serial sections (Figs. 1, 2).

Cells of one typical profile are characterized by less electron-dense cytoplasm than those of smooth muscle cells, and by the presence of abundant mitochondria scattered throughout the whole cytoplasm (Figs. 1–2, 5–8). Cisterns of RER can be observed in the paranuclear cytoplasm, but its cisterns, which often show continuity with SER, rarely show a dilated form (Fig. 8). When they form a gap junction with smooth muscle cells of the main circular layer, they connect their cell bodies to small processes projected from the muscle cells (Figs. 5, 6) or cell bodies of the muscle cells (Figs. 7, 8). Here, it should be emphasized that longer cytoplasmic projections could not be observed in this series of serial sections, of which the total tissue distance was 22.7 μm, as can be seen in Figs. 5–8. They form only a few gap junctions with the cells of the same profile.

A reconstructed model of this cell profile clearly shows that it is an elongated cell showing the same axis as that of the circular muscle cells, with a smooth contour and no branching cytoplasmic processes (Fig. 11). The nucleus shows a flattened and elongated shape. The total area of gap junctions occupies 1.3% of the whole cell surface (Table 1). Gap junctions are mainly distributed over

Figs. 5–8 Electron micrographs of the serial ultrathin sections of a gap-junction-rich cell of the first profile (*C1*). The gap junctions are formed with cytoplasmic projections from smooth muscle cells (**Figs. 5, 6**) and with cell bodies (**Figs. 7, 8**; *arrows*). Note: this cell profile does not project the cytoplasmic process to form gap junctions. Many mitochondria are scattered throughout the whole cytoplasm. Caveolae (**Figs. 7, 8**; *arrowheads*) and a subsurface cistern (**Figs. 6, 8**; *double-headed arrows*) can be seen around the cell membrane. Distances between sections of **Figs. 5–6, 6–7** and **7–8** are 1.0 μm, 14.7 μm and 7.0 μm, respectively. ×23,000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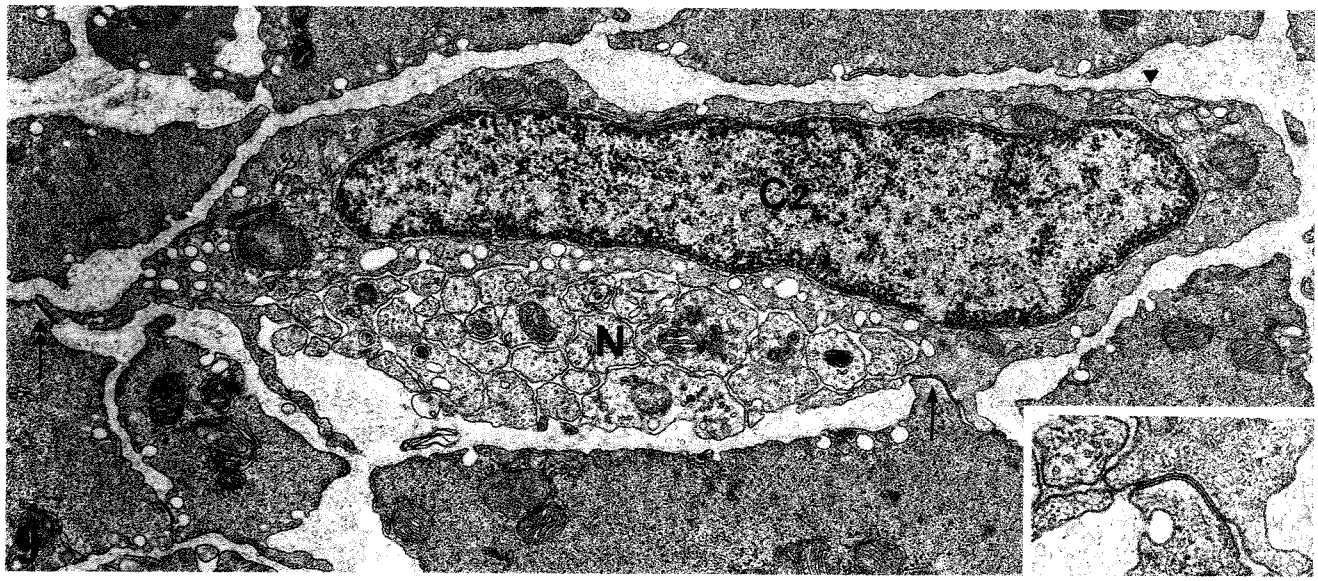
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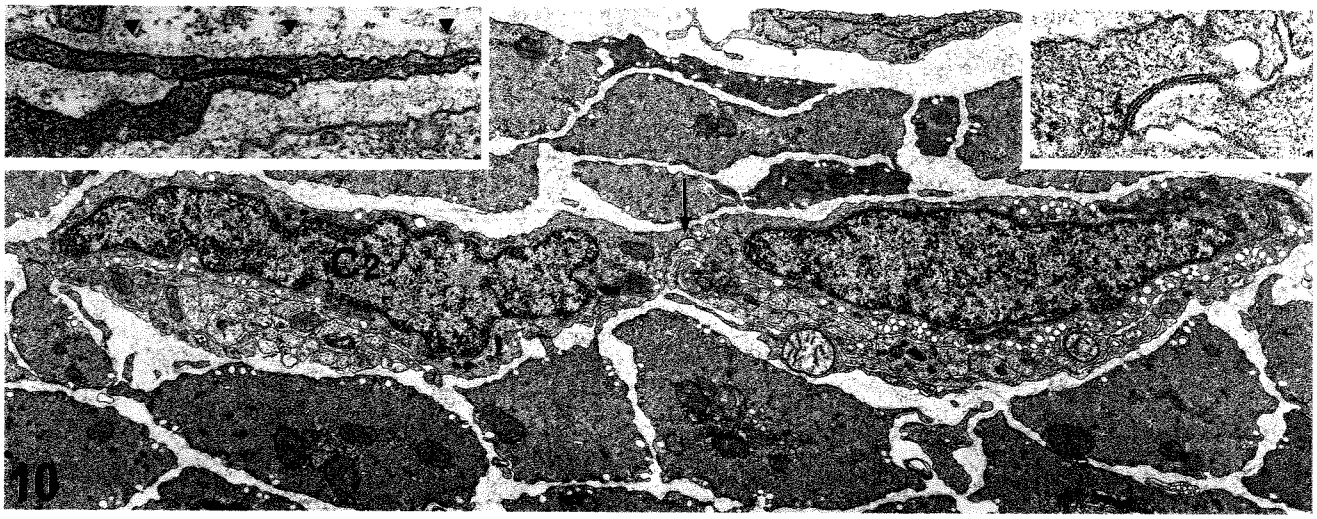
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the cell surface facing the outer main circular muscle layer, with few on the surface toward the inner muscle layer. A large majority of their gap junctions are formed with smooth muscle cells, and only 9.9% of the total area of gap junctions with the cells of the same profile (Table 1).

Cells of another distinct profile are characterized by higher electron-dense cytoplasm and far more conspicuous caveolae (Figs. 1–3, 9, 10) than those of the cell profiles described above. Cisterns of RER often show a dilated form and contain moderately dense materials (Figs. 1–3). They project slender cytoplasmic processes and form gap junctions with the circular muscle cells of the main layer (Fig. 9). They form many gap junctions with cells of the same profile (Fig. 10).

A reconstructed model of this cell profile (Fig. 12) is more flat in form and shows a slender cytoplasmic branch from the cell body. The nucleus shows a flattened, circular shape. Gap junctions are mainly distributed on its cytoplasmic processes and occupy 0.8% of the total cell surface (Table 1). This cell model shows gap junctions with both smooth muscles and cells of the same profile. Of the total area of gap junctions 37.7% is made up of those connecting with the cell of the same profile (Table 1).

Discussion

The present study elucidates further details of the ultrastructural features of the gap junction-rich cells in the rat DMP (Komuro and Seki 1995), which appear to include morphological variations or sub-types of cells. One is characterized by less-electron dense cytoplasm and elongated cell bodies with few processes, with a large total gap junction area – as much as 1.3% of the whole cell surface. It is one-third of the value (4%) of the gap-junction-rich cells of the guinea-pig small intestine (Zhou and Komuro 1992a). These gap junctions mainly connect with the adjacent muscle cells of the main circular layer without forming cytoplasmic branches. On the other hand, the others are characterized by higher electron dense cytoplasm, a number of caveolae and slender cytoplasmic projections that form many gap junctions with the same cell profiles. Their gap junctions occupy 0.8% of the whole cell surface, which is still four times greater than the value (0.2%) of smooth muscle cells of the guinea-pig ileum (Gabella and Blundell 1979, 1981). This cell feature probably results in the formation of their own networks by gap junctions, as well as by connecting with smooth muscle cells.

Although each cell model may not fully represent the complete profile of two putative groups of cells, the observation of cytoplasmic features peculiar to each cell profile in the same section (Figs. 1, 2) seems to rule out the possibility that these differences are simply caused by preparation artifacts such as fixation and sectioning. The latter cells are distinguished from the fibroblast-like cells (Komuro and Seki 1995) that do not have caveolae and a basal lamina. Therefore, it is very likely that there are two morphological varieties of gap-junction-rich cells in the rat DMP region, which may have different functional roles. The existence of two distinctive types of gap-junction-forming cells in the guinea-pig DMP region (Zhou and Komuro 1992a, b) also appears to support such speculation. The cells of the first feature may mainly serve to constitute segmental contraction units by forming cellular networks extending in a circumferential direction, while the cells of the second feature may better contribute to the intercellular communication along the long axis of the small intestine over the muscle bundles of the circular musculature.

Regarding the functional role of ICC, recent studies indicate that ICC in the submuscular plexus (SMP) of the colon (ICC-SMP) are involved in pacemaker activity, while ICC in the DMP of the small intestine (ICC-DMP) primarily function as mediators of neural signals (see reviews by Thuneberg et al. 1995; Komuro et al. 1996; Sanders 1996), even though DMP and SMP have been regarded as equivalent (Rumessen and Thuneberg 1982), and the morphological similarity between ICC-DMP of the mouse (Rumessen et al. 1982) and ICC-SMP of the dog has been pointed out (Berezin et al. 1988).

In the ICC-SMP, an important role of the ICC network connected with gap junctions in the pacemaker function has been demonstrated by the recording of slow waves from the cat colon (Du and Conklin 1989; Conklin and Du 1990), block experiments using heptanol (Huizinga et al. 1988; Serio et al. 1991) or octanol (Farraway et al. 1995) in the dog colon, and by neurobiotin diffusion in the dog colon (Farraway et al. 1995). However, the degree of electrical coupling in the pacemaking region seems to hold an important clue to its activity. It has

◀ **Fig. 9** The second profile of a gap-junction-rich cell (*C2*) is characterized by higher electron-dense cytoplasm and many caveolae. This profile of cell often projects cytoplasmic processes to form gap junctions at the tip of the process (*double-headed arrow*) and at the cell body (*arrow*). A subsurface cistern can be seen (*arrowhead; N* nerve bundle of the DMP). ×21,000. **Inset** Higher magnification of gap junction, indicated by the *arrow*, in a neighboring section to that in Fig. 9. ×40,000

Fig. 10 A neighbouring section of the second profile of cell (*C2*) in Fig. 9. The cell forms a gap junction (*arrow*) with the same profile of cell, which is also characterized by abundant caveolae. ×11,000. **Inset (right)** Higher magnification of the gap junction indicated by the *arrow* in Fig. 10. ×60,000. **Inset (left)** A gap junction between the very thin cytoplasmic processes of the second profile of cells. A basal lamina (*arrowheads*) is also seen. ×54,000

Fig. 11 A three-dimensional model reconstructed from the serial ultrathin sections of the first profile of cell showing its elongated whole-cell shape. Gap junctions are indicated by *pink spots* (formed with smooth muscle cells) and *green spots* (with the same profile of cells). The nucleus is colored in *yellow*. The axis of this cell is almost parallel to that of smooth muscle cell of the inner circular layer colored in *orange* in the upper right corner

Fig. 12 A model of the second profile of cell, which shows a more irregular contour than the first profile of cell. Gap junctions mainly distributed on the cytoplasmic processes are indicated by *pink spots* (formed with smooth muscle cells) and *green spots* (with the same profile of cells). The cell axis differs slightly from the *orange-colored* smooth muscle cell of the inner circular layer indicated in the upper right corner

been suggested that some degree of electrical uncoupling (or high-resistance barrier) within a sinoatrial node (Joyner and Capelle 1986) and between Purkinje and working muscle cells (Tranum-Jessen et al. 1991; Oosthoek et al. 1993) is essential for pace-making activity in the heart, ensuring sufficient source loading.

In this respect, ICC-DMP appear to have a different tissue enivironment from that of ICC-SMP. It has been demonstrated that the gap junction protein Connexin 43 is densely distributed in the circular muscle tissue of the small intestine of several species such as mouse, dog, human (Mikkelsen et al. 1993) and the guinea-pig (Seki et al. 1997), while in contrast only sparse distribution of Connexin 43 is detected in ICC-SMP in the colon. Indeed, the gap-junction-rich cells or probable ICC-DMP of the rat small intestine are not only connected with each other by many large gap junctions, but also are frequently connected with the circular muscle cells (Komuro and Seki 1995; the present observation), which are again interconnected with many gap junctions (Gabella and Blundell 1979, 1981)

In the light of these facts, it is likely that ICC-DMP primarily function as a neuromediator rather than as a pacemaker, since they are also received a rich innervation (Rumessen et al. 1982; Zhou and Komuro 1992b; Komuro and Seki 1995). Such a role of ICC or a neuromediator on nitrergic inhibitory neurotransmission was recently demonstrated in a study using ICC within the muscle layer of the mouse stomach (Burns et al. 1996).

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References

Berezin I, Huizinga JD, Daniel EE (1988) Interstitial cells of Cajal in canine colon: a special communication network at the inner border of the circular muscle. J Comp Neurol 273:42–51

Burns AJ, Lomax AE, Torihashi S, Sanders KM, Ward SM (1996) Interstitial cells of Cajal mediate inhibitory neurotransmission in the stomach. Proc Natl Acad Sci USA 93:12008–12013

Cajal SR (1911) Histologie du système nerveux de l'homme et des vertébrés, vol 2. Maloine, Paris

Conklin JL, Du C (1990) Pathways of slow-wave propagation in proximal colon of cats. Am J Physiol 258:G894–903

Du C, Conklin JL (1989) Origin of slow waves in the isolated proximal colon of the cat. J Auton Nerv Syst 28:167–178

Farraway L, Ball AK, Huizinga JD (1995) Intercellular metabolic coupling in canine colon musculature. Am J Physiol 268:C1492–C1502

Gabella G, Blundell D (1979) Nexuses between the smooth muscle cells of the guinea-pig ileum. J Cell Biol 82:239–247

Gabella G, Blundell D (1981) Gap junctions of the muscle of the small and large intestine. Cell Tissue Res 219:469–488

Huizinga JD, Shin A, Chow E (1988) Electrical coupling and pacemaker activity in colonic smooth muscle. Am J Physiol 255:C653–C660

Joyner RW, Capelle FJL (1986) Propagation through electrically coupled cells. Biophys J 50:1157–1164

Komuro T, Seki K (1995) Fine structural study of interstitial cells associated with the deep muscular plexus of the rat small intestine, with special reference to the intestinal pacemaker cells. Cell Tissue Res 282:129–134

Komuro T, Tokui K, Zhou DS (1996) Identification of the interstitial cells of Cajal. Histol Histopathol 11:769–786

Mikkelsen HB, Huizinga JD, Thuneberg L, Rumessen JJ (1993) Immunohistochemical localization of a gap junction protein (connexin 43) in the muscularis externa of murine, canine, and human intestine. Cell Tissue Res 274:249–256

Oosthoek PW, Virágh S, Mayen AEM, Kempen MJA van, Lamers WH, Moorman FM (1993) Immunohistochemical delineation of the conduction system. I. The sinoatrial node. Circ Res 73: 473–481

Rumessen JJ, Thuneberg L (1982) Plexus muscularis profundus and associated interstitial cells. I. Light microscopical studies of mouse small intestine. Anat Rec 203:115–127

Rumessen JJ, Thuneberg L, Mikkensen HB (1982) Plexus muscularis profundus and associated interstitial cells. II. Ultrastructure studies of mouse small intestine. Anat Rec 203:129–146

Sanders KM (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. Gastroenterology 111:492–515

Seki K, Zhou DS, Komuro T (1997) Immunohistochemical study of the *c-kit* expressing cells and connexin 43 in the guinea-pig digestive tract. J Auton Nerv Syst (in press)

Serio R, Barajas-Lopez C, Daniel EE, Berezin I, Huizinga JD (1991) Slow-wave activity in colon: role of network of submucosal interstitial cells of Cajal. Am J Physiol 260:G636–645

Thuneberg L (1982) Interstitial cells of Cajal: intestinal pacemaker cells? Adv Anat Embryol Cell Biol 71:1–130

Thuneberg L, Rumessen JJ, Mikkelsen HB, Peters S, Jessen H (1995) Structural aspects of intestitial cells of Cajal as intestinal pacemaker cells. In: Huizinga JD (ed) Pacemaker activity and intercellular communication. CRC Press, Boca Raton, pp 193–222

Torihashi S, Kobayashi S, Gerthoffer WT, Sanders KM (1993) Interstitial cells in deep muscular plexus of canine small intestine may be specialized smooth muscle cells. Am J Physiol 265:G638–645

Tranum-Jensen J, Wilde AAM, Vermeulen T, Jense MJ (1991) Morphology of electrophysiologically identified junctions between Purkinje fibers and ventricular muscle in rabbit and pig hearts. Circ Res 69:429–437

Zhou DS, Komuro T (1992a) Interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine, with special reference to the interstitial cells of Cajal. Cell Tissue Res 268:205–216

Zhou DS, Komuro T (1992b) The cellular network of interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine. Anat Embryol 186:519–527

Cell Tissue Res (1998) 293:277–284
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REGULAR ARTICLE

Kazuhide Horiguchi · Terumasa Komuro

Ultrastructural characterization of interstitial cells of Cajal in the rat small intestine using control and *Ws/Ws* mutant rats

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Abstract Interstitial cells in the myenteric plexus and the deep muscular plexus of the small intestine of the c-*kit* mutant rats (*Ws/Ws*) and their normal siblings (+/+) were studied. c-Kit immunoreactivity was detected in two regions corresponding to the myenteric plexus and the deep muscular plexus in the jejunum of +/+ rats, while no immunoreactivity was detected in *Ws/Ws* rats. Using electron microscopy, two types of gap junction-forming interstitial cells were found in association with the myenteric plexus in +/+ rats: one type characterized by a typical fibroblastic ultrastructure, and the other characterized by numerous mitochondria and less electron-dense cytoplasm. Since the latter were greatly reduced in *Ws/Ws* rats, it was suggested that these cells correspond to c-*kit*-expressing cells, i.e. interstitial cells of Cajal in the myenteric plexus region. In contrast, two types of interstitial cells in the region of the deep muscular plexus were observed with no difference between +/+ and *Ws/Ws* rats. Probable interstitial cells of Cajal in this region were characterized by a basal lamina and numerous caveolae as well as large gap junctions that interconnect with each other and with the smooth muscle cells. We concluded that interstitial cells of Cajal in the rat intestine are heterogeneous in ultrastructure, c-*kit* dependency in the cell maturation, and functional role.

Key words Interstitial cells of Cajal (ICC) · c-Kit · Small intestine pacemaker · Morphology · Rat (*Ws/Ws*)

Introduction

Recent studies indicate that the cells expressing c-Kit receptor in the mouse small intestine correspond to interstitial cells of Cajal (ICC; Cajal 1911), which have been proposed as the pacemakers of intestinal peristalsis (Thuneberg 1982), by demonstrating that the postnatal blockade of the receptor with its antibody (Maeda et al. 1992; Torihashi et al. 1995) or its genetic defect (Ward et al. 1994; Huizinga et al. 1995) results in a loss of pacemaker activity or electrical slow waves in the intestine. Abnormalities in the ileal movement and pyloric sphincter function have also been reported in *Ws/Ws* rats in which the tyrosine kinase activity of c-Kit is severly impaired (Isozaki et al. 1995). The gene product of c-*kit* is a receptor tyrosine kinase and is encoded by the mouse *W* locus and the rat *Ws* locus (Chabot et al. 1988; Tsujimura et al. 1991). The extracellular domain contains the receptor for stem cell factor (SCF), the natural ligands for c-Kit receptors, and the cytoplasmic domain conveys tyrosine kinase activity. SCF is encoded by the mouse *Sl* locus (Williams et al. 1990; Zsebo et al. 1990).

Isozaki et al. (1995) have reported that a small number of c-*kit* expressing cells are present in the small intestine in contrast to the complete absence of those cells in the stomach of *Ws/Ws* rats, and have suggested that the development of ICC in the intestine could be less dependent on the c-Kit-SCF system than their development in the stomach. They have also suggested the possibility of the presence of c-*kit*-negative ICC in the gastrointestinal tract.

A most interesting question is whether different classes of ICC constitute morphological and functional subtypes of ICC or represent different cell lineages, since Thuneberg (1982) has classified four types of ICC for the first time based on their tissue locations and ultrastructural features. Recently, Burns et al. (1997) classified six types of ICC in the guinea-pig gastrointestinal tract by using c-Kit immunohistochemistry.

The present study intends to characterize the ultrastructural features of ICC in the deep muscular plexus (ICC-DMP) and Auerbach's (myenteric) plexus (ICC-AP), corresponding to c-*kit* expressing cells in each location by a comparison of *Ws/Ws* rats and their normal siblings. Although similar studies using either W/W^v mouse, which has a point mutation of c-*kit* (Malysz et al. 1996), or Sl/Sl^d mouse, which has a mutation in the ligand for c-Kit (Ward et al. 1995), have reported differences between

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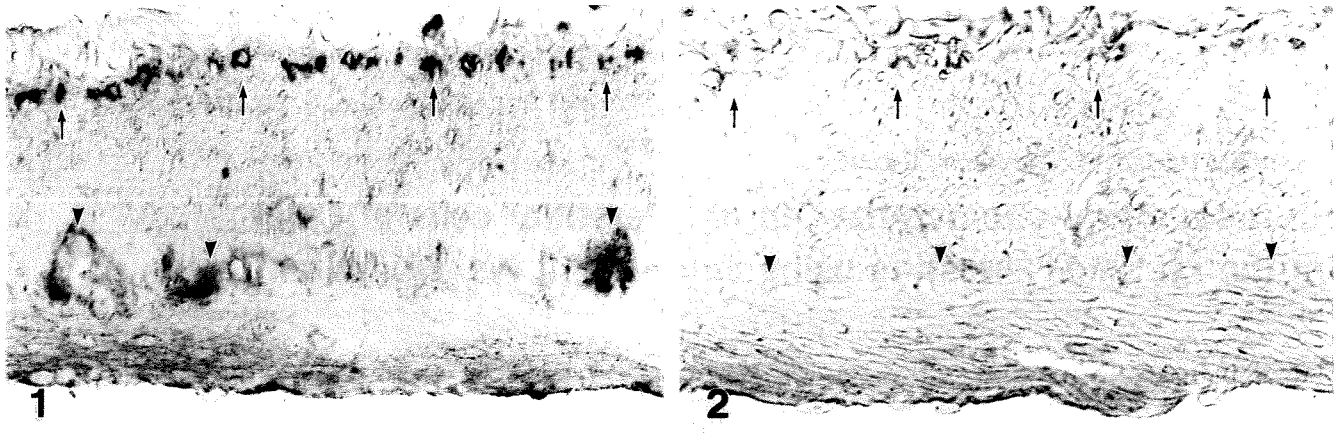


Fig. 1 Dense immunoreactive deposits to anti-c-Kit antibody are observed in the region corresponding to the DMP (*arrows*) in the control +/+ rat small intestine. Moderate immunoreactivity is also seen in the region of the myenteric plexus (*arrowheads*). ×320

Fig. 2 No immunoreactivity to anti-c-Kit antibody is observed in the regions corresponding to the DMP (*arrows*) and to the myenteric plexus (*arrowheads*) in *Ws/Ws* rat small intestine. ×320

ICC-DMP and ICC-AP on c-Kit dependency in cell maturation, their ultrastructures have not been thoroughly examined. Besides, it has been known that the DMP region of both rats (Komuro and Seki 1995; Seki and Komuro 1997) and guinea-pigs (Zhou and Komuro 1992a,b) and the myenteric region of guinea-pigs (Komuro et al. 1996) contain more than one type of interstitial cells forming close contacts with nerve terminals and gap junctions with smooth muscles which are believed to be characteristic features of ICC. Thus, a critical examination of those cells seems to be necessary to further clarify the cytological nature of the non-neural regulatory system, including ICC, in the gut motility. A preliminary account of this study has been published elsewhere (Horiguchi et al. 1995).

Materials and methods

Immunohistochemistry

Seven homozygous *Ws/Ws* mutant rats and sibling control +/+ rats (aged 4–8 weeks) were used. Under terminal anesthesia with ether, short segments of proximal jejunum were removed from these animals and frozen in liquid nitrogen in OCT compound (Tissue Tek). Cryostat sections were cut at a thickness of 10 μm and collected on gelatin-coated slides. The specimens were fixed with acetone for 10 min at room temperature, rinsed in phosphate-buffered saline (PBS) several times, and incubated with 4% Block Ace solution (Dainippon Seiyaku) for 20 min at room temperature to prevent nonspecific antibody binding. Then specimens were incubated overnight at 4°C with a primary antiserum against human c-Kit protein (C-19; SantaCruz Biotechnology, rabbit polyclonal), at a dilution ratio of 1:50 in PBS-azide. After washing in PBS, the specimens were further incubated overnight at 4°C with peroxidase-conjugated secondary antibodies (DAKO, swine anti-rabbit IgG) at a dilution ratio of 1:50. Horseradish peroxidase reaction was developed in 50 ml 0.1 M TRIS-HCl buffer (pH 7.4) solution containing 6 mg 4-chloro-1-naphtol (Sigma) and 8 μg 30% H_2O_2.

Electron microscopy

Short segments of proximal jejunum were removed with the animals under ether anesthesia, and placed in a fixative containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h at 4°C. The specimens were rinsed in the same buffer and post-fixed in 1% osmium tetroxide for 2 h at 4°C. The specimens were then rinsed in distilled water, block-stained with saturated uranyl acetate solution for 3 h, dehydrated in a graded series of ethyl alcohols and embedded in Epon epoxy resin. Ultrathin sections were cut using a Reichert microtome and double-stained with uranyl acetate and lead tartrate for observation under a JEM 1200EX II electron microscope.

For counting the cell number of interstitial cells in the myenteric region, only cell profiles with a nucleus were counted along the border of the circular muscle cells on montages of electron micrographs of the control +/+ and *Ws/Ws* rats.

Results

c-Kit immunohistochemistry

Immunoreactivity to anti-c-Kit antibody was clearly observed in two regions corresponding to the DMP and the myenteric plexus in the jejunum of +/+ control rats (Fig. 1). In contrast to the control rats, no immunoreactivity was detectable in the myenteric plexus region or the DMP region of *Ws/Ws* rats (Fig. 2).

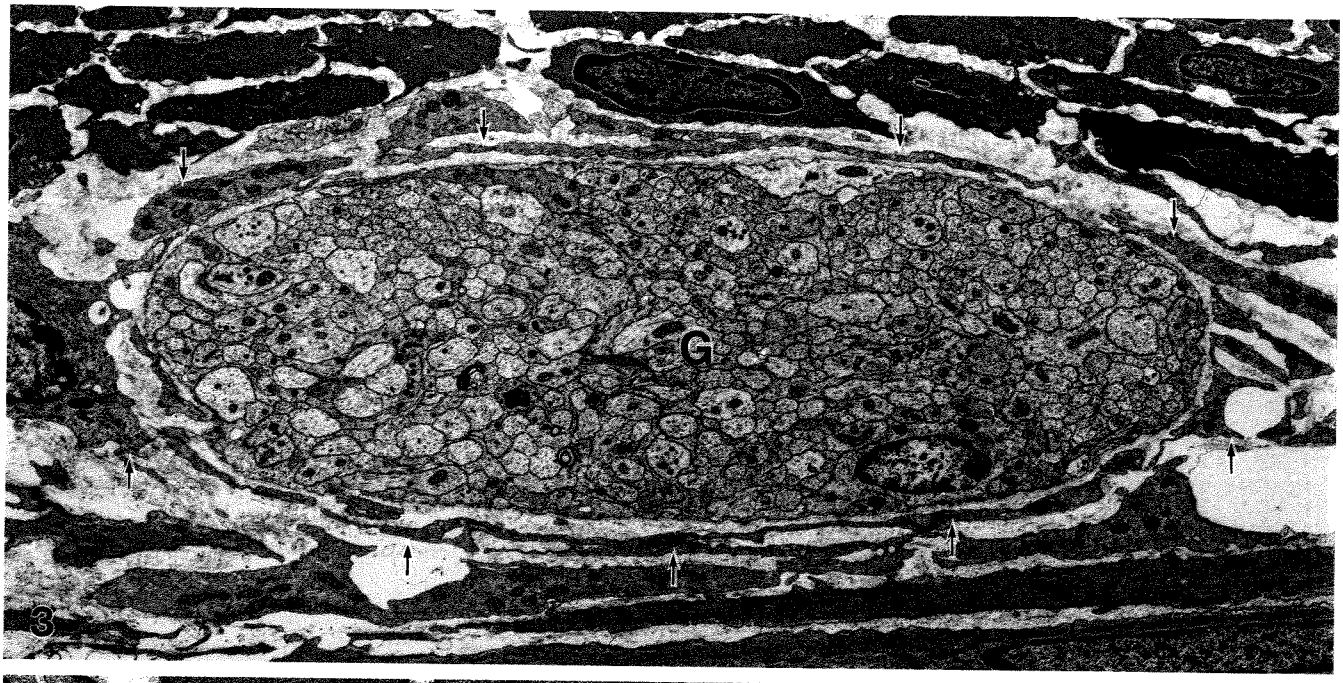
Interstitial cells in the myenteric region

Control +/+ rats. Many interstitial cells are observed in this region so that the myenteric ganglia are usually sur-

Fig. 3 Electron micrograph showing a myenteric ganglion (*G*) surrounded by a nearly complete sheath consisting of interstitial cells and their processes (*arrows*) in the control +/+ rat small intestine. ×7000

Fig. 4 A fibroblast-like cell located in the myenteric plexus region in the control +/+ rat. Well-developed RER is conspicuous and its cisterns contain moderate electron-dense material. It forms a small gap junction with smooth muscle cells (*arrow*). ×20000. *Inset* Higher magnification of the gap junction indicated by the *arrow*. ×80000

Fig. 5 ICC-AP observed in +/+ rat, which is characterized by many mitochondria and less electron-dense cytoplasm. ×12000. *Inset* A gap junction between slender cytoplasmic processes of ICC-AP. Note, there is no basal lamina around the cell membrane. ×96000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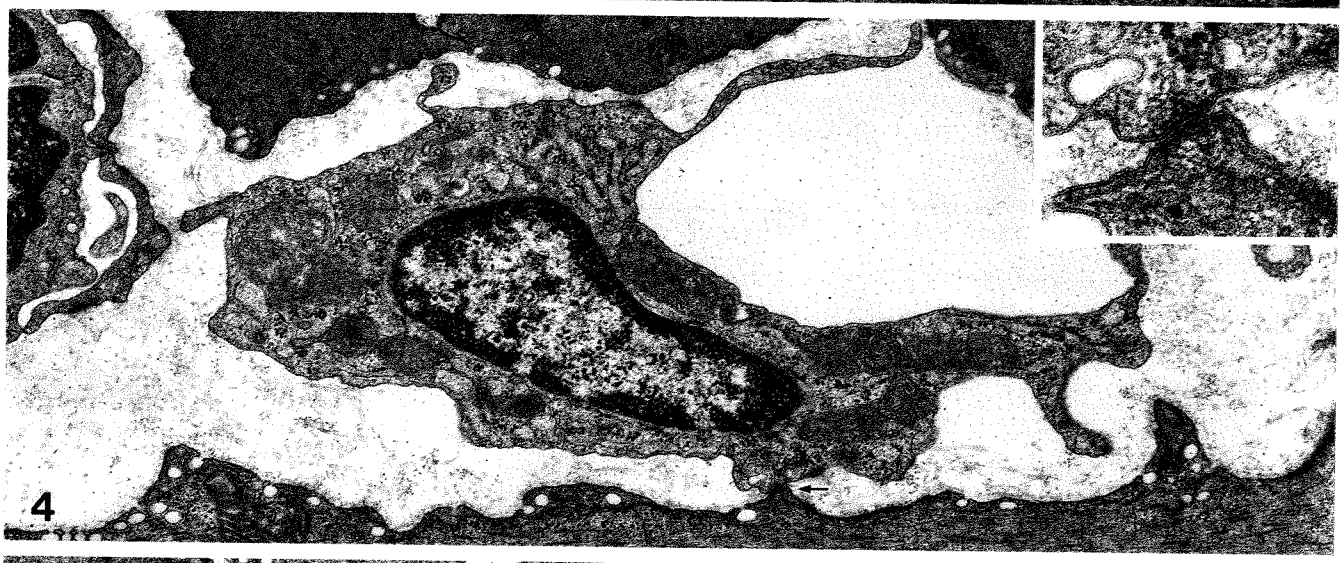
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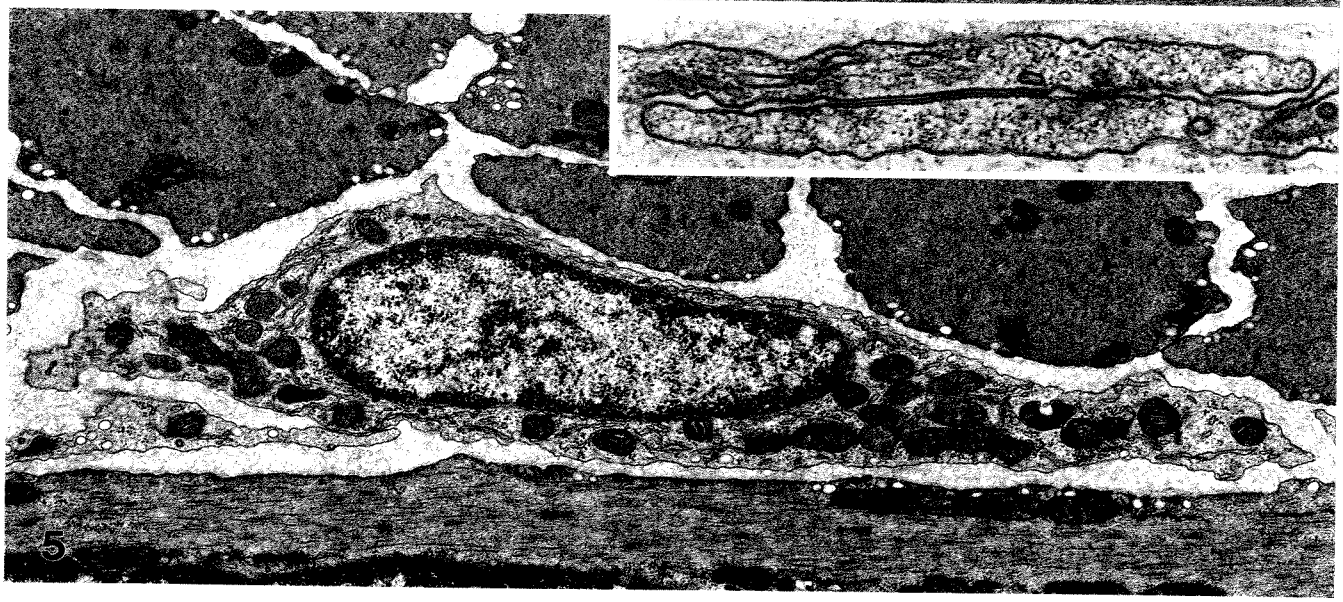
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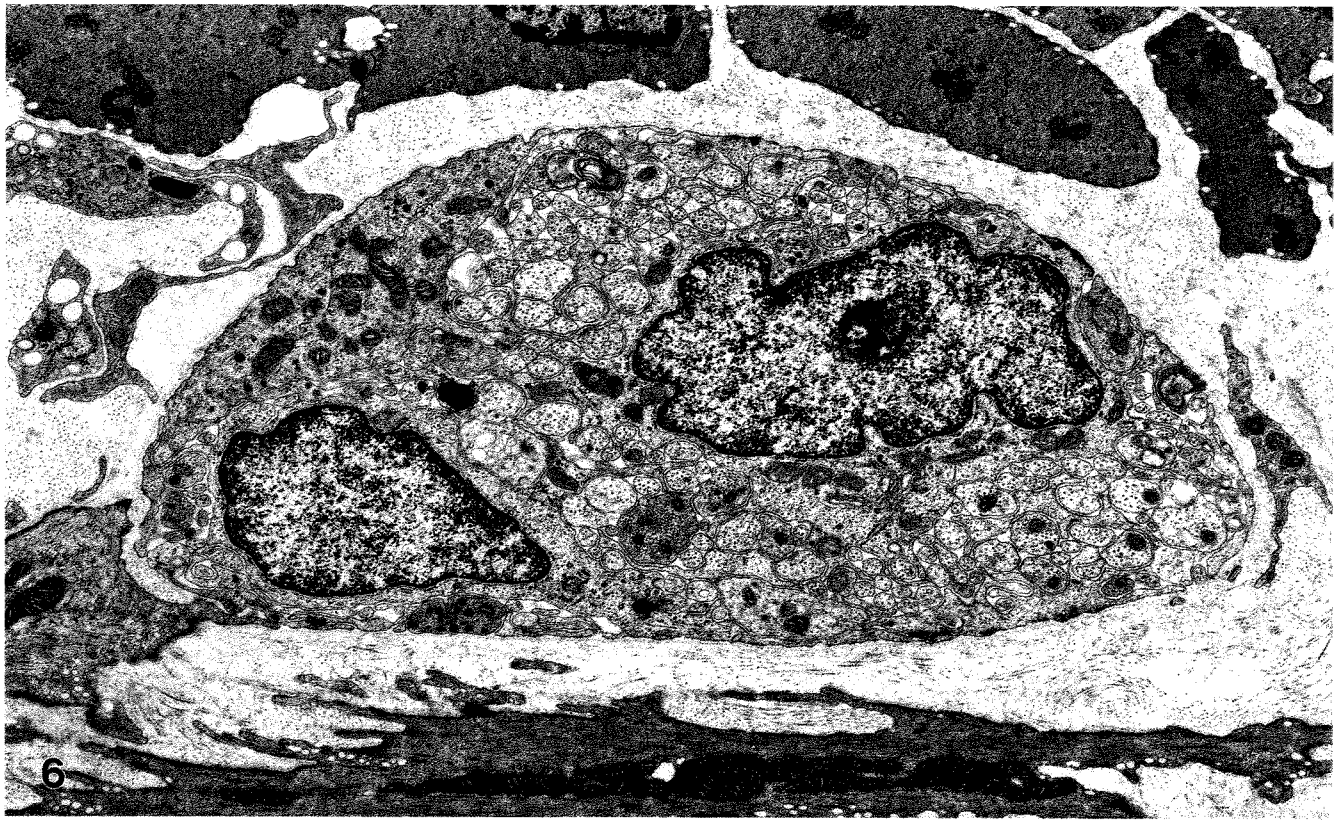


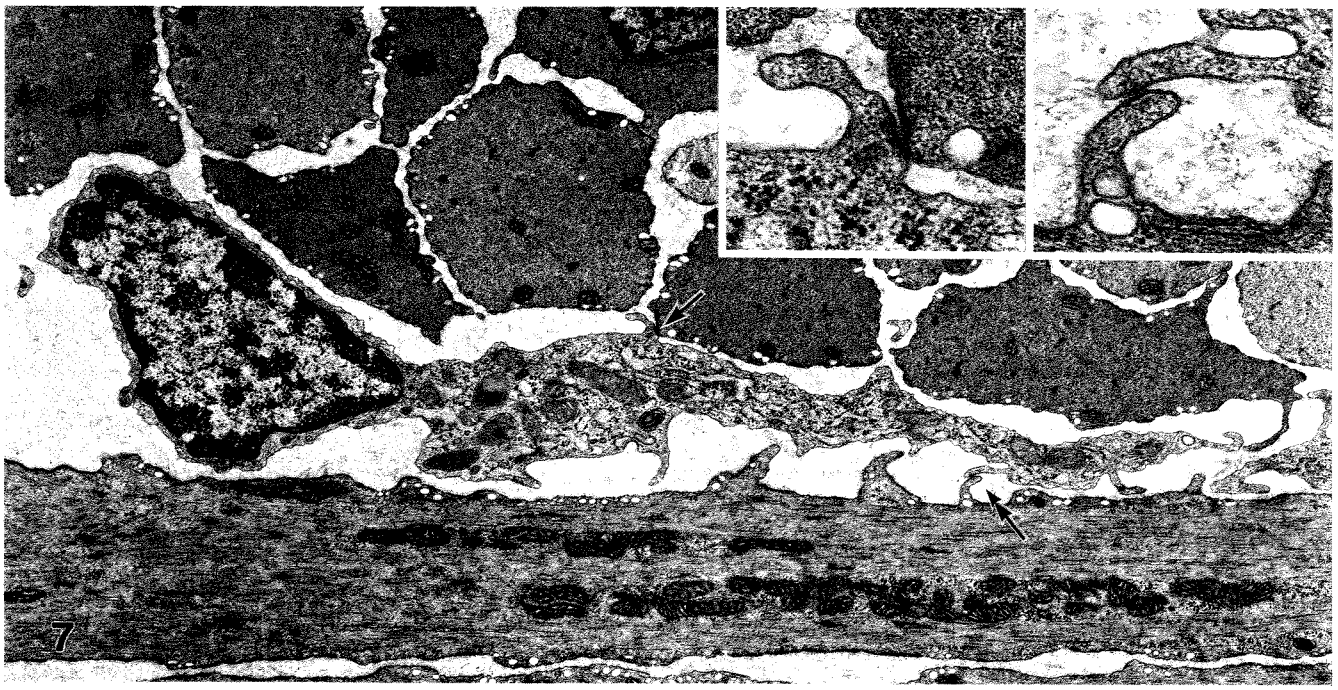
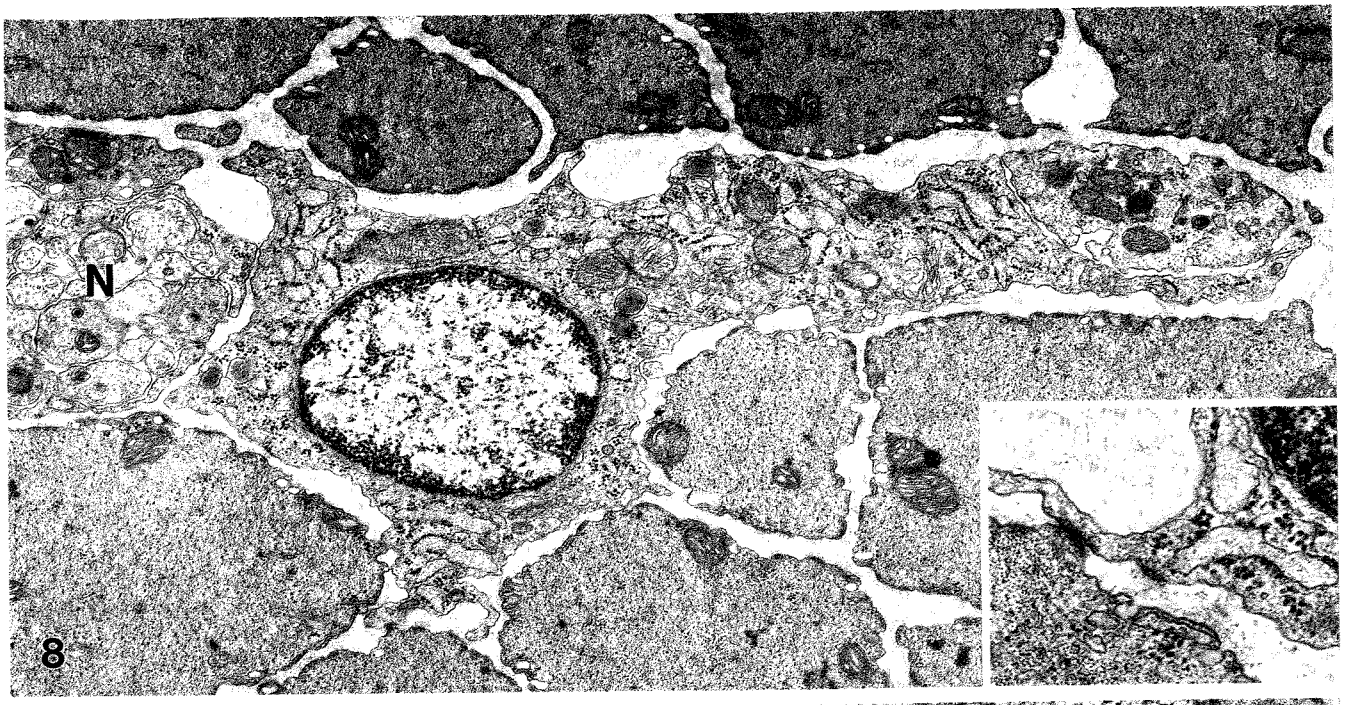


Fig. 6 A myenteric ganglion in *Ws/Ws* rat, which is accompanied by a few processes of interstitial cells. ×11000

Fig. 7 A fibroblast-like cell located in the myenteric region of *Ws/Ws* rat. It forms small gap junctions with the muscle cells of both circular (*arrow*) and longitudinal (*double-headed arrow*) layers. ×10000. *Inset* Higher magnification of the gap junction indicated by an *arrow* and a *double-headed arrow*. ×52000

rounded by an almost continuous sheet of their cytoplasmic processes(Fig. 3). Two types of the interstitial cells were distinguished by their ultrastructural features.

Cells of the first type show features of typical fibroblasts (Fig. 4). Their nuclei are elongated and have condensed chromatin. They have well-developed rough endoplasmic reticulum (RER) of which cisterns are often dilated, and contain moderately dense material. Free ribo-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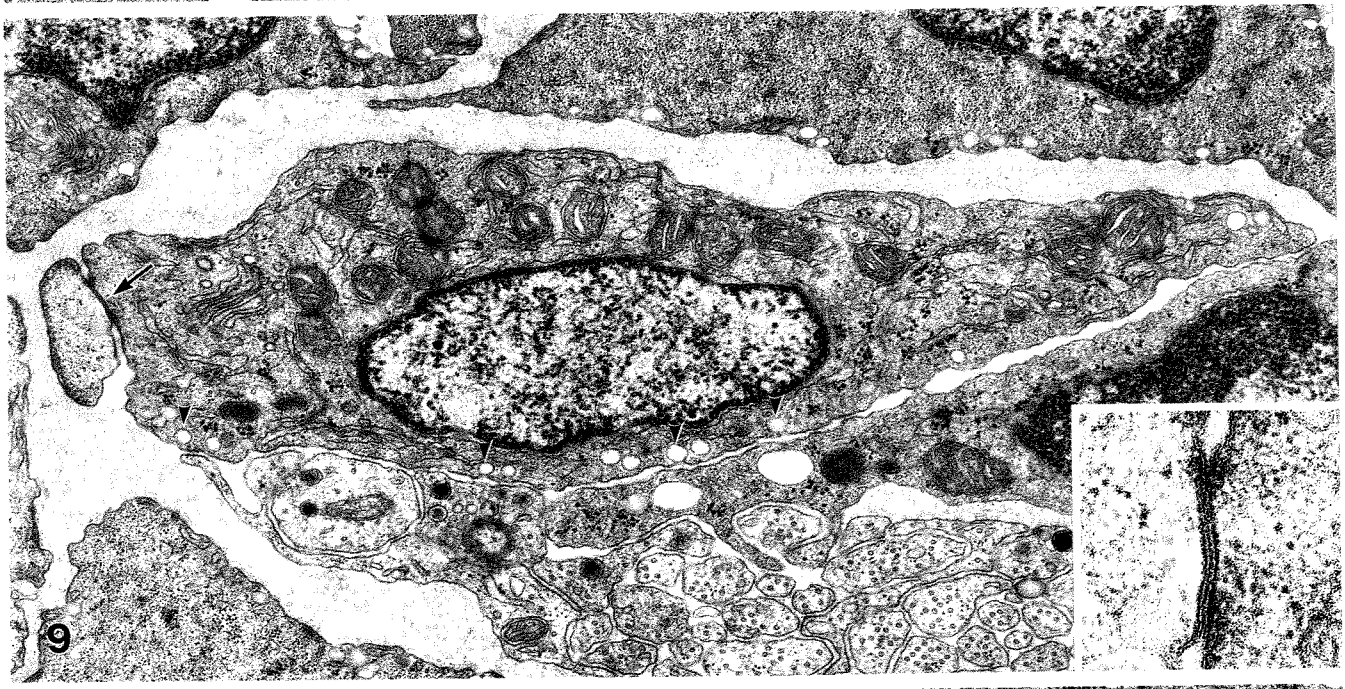
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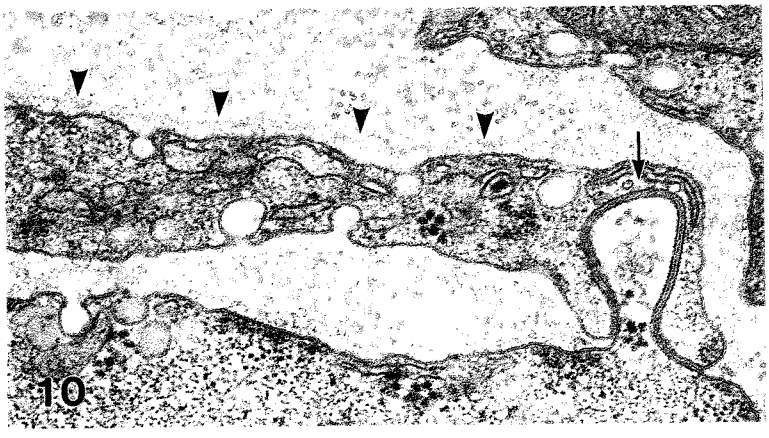
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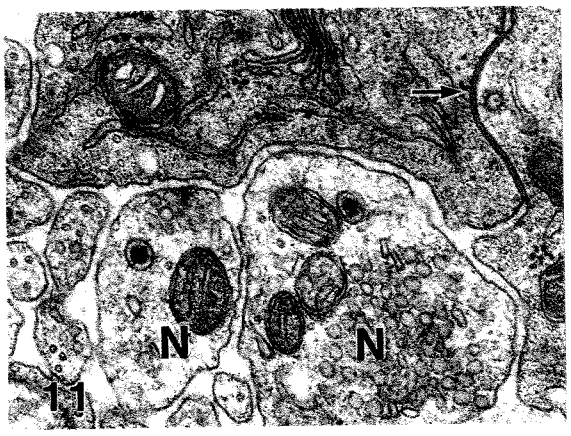
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somes are dispersed in the cytoplasm. Golgi apparatus can be seen. There is no basal lamina or caveolae. Microtubules, intermediate filaments, and thin filaments are indistinct. They often connect with smooth muscle cells by small gap junctions (Fig. 4, inset).

Cells of another type are characterized by numerous mitochondria and less electron-dense cytoplasm (Fig. 5). Golgi apparatus and smooth endoplasmic reticulum are well-developed. RER is also seen, but its cisterns rarely show a dilated form. Caveolae and basal lamina are not observed. They form gap junctions with each other (Fig. 5, inset) and with smooth muscle cells.

Ws/Ws rats. Interstitial cells and their processes are relatively sparse in the myenteric region of *Ws/Ws* rats (Fig. 6). Cells of the first type are observed without noticable change, like the +/+ rats (Fig. 7). It is worth noting that even a single fibroblast-like cell forms gap junctions with smooth muscle cells of both circular and longitudinal type in a single section (Fig. 7, inset). However, the second type of cells is greatly reduced in *Ws/Ws* rats. Examination of the number of interstitial cells on the montage micrographs reveals that 21 cells of the second type are counted along a length of 468 cross-sectioned smooth muscle cells bordering the myenteric plexus in +/+ rats, in contrast to only 2 cells observed along a length of 573 muscle cells in *Ws/Ws* rats. On the other hand, 16 and 23 cells of the first type are counted on the same montages in +/+ and *Ws/Ws* rats, respectively.

Interstitial cells in the DMP region

Deep muscular plexus (DMP) is located between two subdivisions of the circular muscle layer: an inner thin and an outer thick layer. Two types of interstitial cells are also recognized in this region. In contrast to those of the myenteric region, both types of interstitial cells in the DMP show no difference between +/+ and *Ws/Ws* rats. Thus, the following description applies only to the observation in the *Ws/Ws* rats.

Cells of the first type show features similar to those of the typical fibroblast. They are characterized by well-developed RER whose cisterns contain moderately dense material (Fig. 8). Golgi apparatus and free ribosomes are also observed. Cytoskeletal elements are inconspicuous in the cytoplasm. There is no basal lamina or caveola. They are often closely associated with nerve varicosities (Fig. 8) and form a few small gap junctions with the smooth muscle cells (Fig. 8, inset).

Cells of the second type are characterized by a number of mitochondria. The most conspicuous feature of this type of cell is the presence of large gap junctions connected with smooth muscle cells of the main layer and with one another (Figs. 9–11). Golgi apparatus, free ribosomes, and RER are also observed in the cytoplasm. However, cisterns of RER in this cell type rarely show a dilated form. Subsurface cisterns can be seen beneath the cell membrane. A continuous basal lamina and caveolae are clearly observed (Fig. 10). Microtubules, intermediate filaments, and thin filaments are also seen. These cells show close contacts with nerve varicosities (Fig. 11).

Discussion

The present study reveals that one type of gap junction-forming interstitial cell, characterized by low electron-dense cytoplasm, abundant mitochondria, no basal lamina and no caveolae in the myenteric region, is greatly reduced in the *Ws/Ws* rat small intestine, in contrast to fibroblast-like cells, which remain unchanged. Since the great reduction of their cell number is consistent with the loss of the immunoreactivity to anti-c-Kit antibody in the myenteric region of *Ws/Ws* rats, it can be concluded that those mitochondria-rich cells represent the c-*kit* expressing cells in the myenteric region, or ICC-AP in the rats. This type of cell seems to correspond to the mitochondria-rich cells previously described in the small intestine of the normal Wistar rats, which demonstrate a well-demarcated cell body and a few long slender cytoplasmic processes under the scanning electron microscope (Komuro 1989). This means that the present observation confirms that ICC-AP of the rat small intestine do not show any myoid features such as basal lamina or caveolae, and that they differ ultrastructurally from ICC-AP of the mouse intestine, which have been described as showing electron-dense cytoplasm, patch basal lamina and numerous caveolae (Thuneberg 1982; Huizinga et al. 1995; Ward et al. 1994,1995). Their ultrastructure also differs from those of ICC within the circular muscle layer of the rat stomach, which are characterized by the presence of many caveolae and electron-dense cytoplasm, as well as by large gap junctions and abundant mitochondria (Ishikawa et al. 1997). The marked increase in number of fibroblast-like cells reported in W/W^v mouse (Malysz et al. 1996) was not observed in *Ws/Ws* rats.

In contrast to the cells in the myenteric region, two types of interstitial cells in the DMP region were observed in *Ws/Ws* rats similar to those in their normal siblings, in spite of the absence of immunoreactivity to c-Kit. This fact makes it difficult to deduce which type of cells corresponds to c-*kit* expressing cells or ICC-DMP. However,

◀ **Fig. 8** A fibroblast-like cell located in the DMP region of *Ws/Ws* rat. Well-developed RER are conspicuous in the cytoplasm (*N* nerve bundles). ×17,000. *Inset* Higher magnification of the small gap junction between the cell of this type and smooth muscle cell. ×40000

Fig. 9 ICC-DMP of *Ws/Ws* rat, characterized by many mitochondria, caveolae (*arrowheads*), and gap junction (*arrow*) with a muscle cell. ×27000. *Inset* Higher magnification of the gap junction indicated by the *arrow*

Fig. 10 A cytoplasmic process of ICC-DMP forming a gap junction with a circular muscle cell (*arrow*). A distinct basal lamina is indicated by *arrowheads*. ×65000

Fig. 11 Axon terminals (*N*) containing synaptic vesicles closely associated with ICC-DMP of *Ws/Ws* rat. An *arrow* indicates a gap junction in the same type of cells. ×36000

fibroblast-like cells in both myeteric and DMP regions are characterized by exactly the same ultrastructural features, and are observed in *Ws/Ws* the same as in control animals. Thus, it can be speculated that another cell type characterized by a basal lamina, caveolae and many large gap junctions in this region corresponds to c-*kit* expressing cells there, or ICC-DMP in the rat small intestine.

The unchanged observation of ICC-DMP in *Ws/Ws* rats appears to indicate that they are able to develop and mature independently from the c-kit-SCF system, as suggested in the studies of W/W^v mouse (Malysz et al. 1996). Although it was reported that development of ICC-DMP showed different degrees of lesion, depending on timing and the number of postnatal injections of anti-c-Kit protein (ACK2) to BALB/c mouse (Torihashi et al. 1995), a normal distribution of ICC-DMP was also observed in Sl/Sl^d mouse (Ward et al. 1995).

Regarding the functional role of ICC, it is speculated that ICC-DMP are not involved in the generation of pacemaker activity, since they are observed in W/W^v mouse (Malysz et al. 1996) and Sl/Sl^d mouse (Ward et al. 1995), which lack normal activity of intestinal contraction and slow waves. The developmental study also revealed that electrical slow waves develop after the formation of an ICC-AP network, but before the appearance of ICC-DMP (Torihashi et al. 1997). The present observation of ICC-DMP in *Ws/Ws* rats, which have showed apparent abnormal activity in ileal contraction (Isozaki et al. 1995), seems to confirm this assumption, while ICC-AP are very likely to play an important role in the pacemaking function. Since ICC-DMP of the rat intestine have rich innervation and form many gap junctions with each other and with smooth muscle cells (Komuro and Seki 1995; Seki and Komuro 1997; the present study), they appear to function as a mediator of nerve signals to the smooth muscle, as suggested repeatedly in previous studies (Thuneberg 1982; Komuro et al. 1996; Sanders 1996). This assumption is compatible with the conclusion that ICC-DMP of the small intestine were most densely coupled with each other and with the circular muscle tissue via gap junctions, in comparison with the muscular tissue in the stomach and colon, by the immunohistochemical observation of c-*kit* expressing cells and gap junction protein (connexin 43) in the guinea pig gastrointestinal tract (Seki et al. 1998).

On the other hand, the present study demonstrates that both DMP and myenteric regions of the rat intestine contain fibroblast-like cells forming close contact with nerve varicosities and gap junctions with smooth muscle cells without apparent dependency on c-*kit*. Their cellular connection to both circular and longitudinal muscle cells by gap junctions is observed even in *Ws/Ws* rats, similar to those observed in normal Wistar rats (Komuro 1989). The gap junction-forming fibroblast-like cells were also observed within the circular muscle layer of the *Ws/Ws* rat stomach (Ishikawa et al. 1997). It is very likely that those fibroblast-like cells mediate electrical signals to muscle cells, though supporting data have not been available so far.

The cytological and functional heterogeneity of gap junction-forming interstitial cells, including ICC located in different tissue layers of different levels of digestive tract, is an important issue for future studies for a better understanding of the peculiar contractile activity of each organ.

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References

Burns AJ, Herbert TM, Ward SM, Sanders KM (1997) Interstitial cells of Cajal in the guinea-pig gastrointestinal tract as revealed by c-Kit immunohistochemistry. Cell Tissue Res 290:11–20

Cajal SR (1911) Histologie du systéme nerveux de l'homme et des vertébrés. Tome 2. Maloine, Paris

Chabot B, Stephenson DA, Chapman VM, Besmer P, Bernstein A (1988) The proto-oncogene c-*kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. Nature 335:88–89

Horiguchi K, Zhou DS, Seki K, Komuro T, Hirota S, Kitamura Y, Nomura S (1995) Morphological analysis of c-*kit* expressing cells, with reference to interstitial cells of Cajal. J Smooth Muscle Res 31:303–305

Huizinga JD, Thuneberg L, Klüppel M, Malysz J, Mikkelsen HB, Bernstein A (1995) *W*/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature 373:347–349

Ishikawa K, Komuro T, Hirota S, Kitamura Y (1997) Ultrastructural identification of the *c-kit*-expressing interstitial cells in the rat stomach: a comparison of control and *Ws/Ws* mutant rats. Cell Tissue Res 289:137–143

Isozaki K, Hirota S, Nakama A, Miyagawa J, Shinomura Y, Xu Z, Nomura S, Kitamura Y (1995) Disturbed intestinal movement, bile reflux to the stomach, and deficiency of c-*kit* expressing cells in *Ws/Ws* mutant rats. Gastroenterology 109:456–464

Komuro T (1989) Three-dimensional observation of the fibroblast-like cells associated with the rat myenteric plexus, with special reference to the interstitial cells of Cajal. Cell Tissue Res 255:343–351

Komuro T, Seki K (1995) Fine structural study of interstitial cells associated with the deep muscular plexus of the rat small intestine, with special reference to the intestinal pacemaker cells. Cell Tissue Res 282:129–134

Komuro T, Tokui K, Zhou DS (1996) Identification of the interstitial cells of Cajal. Histol Histopathol 11:769–786

Maeda H, Yamagata A, Nishikawa S, Yoshinaga K, Kobayashi S, Nishi K, Nishikawa S (1992) Requirement of c-*kit* for development of intestinal pacemaker system. Development 116:369–375

Malysz J, Thuneberg L, Mikkelsen HB, Huizinga JD (1996) Action potential generation in the small intestine of *W* mutant mice that lack interstitial cells of Cajal. Am J Physiol 271:G387–399

Sanders KM (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. Gastroenterology 111:492–515

Seki K, Komuro T (1997) Further observation of the gap junction-rich cells in the deep muscular plexus of the rat small intestine. Anat Embryol 197:135–141

Seki K, Zhou DS, Komuro T (1998) Immunohistochemical study of the c-*kit* expressing cells and connexin 43 in the guinea-pig digestive tract. J Auton Nerv Syst 68:182–187.

Thuneberg L (1982) Interstitial cells of Cajal: intestinal pacemaker cells? Adv Anat Embyol Cell Biol 71:1–130

Torihashi S, Ward SM, Nishikawa S, Nishi K, Kobayashi S, Sanders KM (1995) c-*kit* dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res 280:97–111

Tsujimura T, Hirota S, Nomura S, Niwa Y, Yamazaki M, Tono T, Morii E, Kim HM, Kondo K, Nishimune Y, Kitamura Y (1991) Characterization of *Ws* mutant allele of rats: a 12-base deletion in tyrosine kinase domain of c-*kit* gene. Blood 78:1942–1946

Ward SM, Burns AJ, Torihashi S, Sanders KM (1994) Mutation of the proto-oncogene c-*kit* blocks development of interstitial cells and electrical rhythmicity in murine intestine. J Physiol (Lond) 480:91–97

Ward SM, Burns AJ, Torihashi S, Harney SC, Sanders KM (1995) Impaired development of interstitial cells and intestinal electrical rhythmicity in *steel* mutants. Am J Physiol 269:C1577–1585

Williams DE, Eisenman J, Baird A, Rauch C, van Ness K, March CJ, Park LS, Martin U, Mochizuki DY, Boswell HS, Burgess GS, Cosman D, Lyman DS (1990) Identification of a ligand for the c-*kit* proto-oncogene. Cell 63:167–174

Zhou DS, Komuro T (1992a) Interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine, with special reference to the interstitial cells of Cajal. Cell Tissue Res 268:205–216

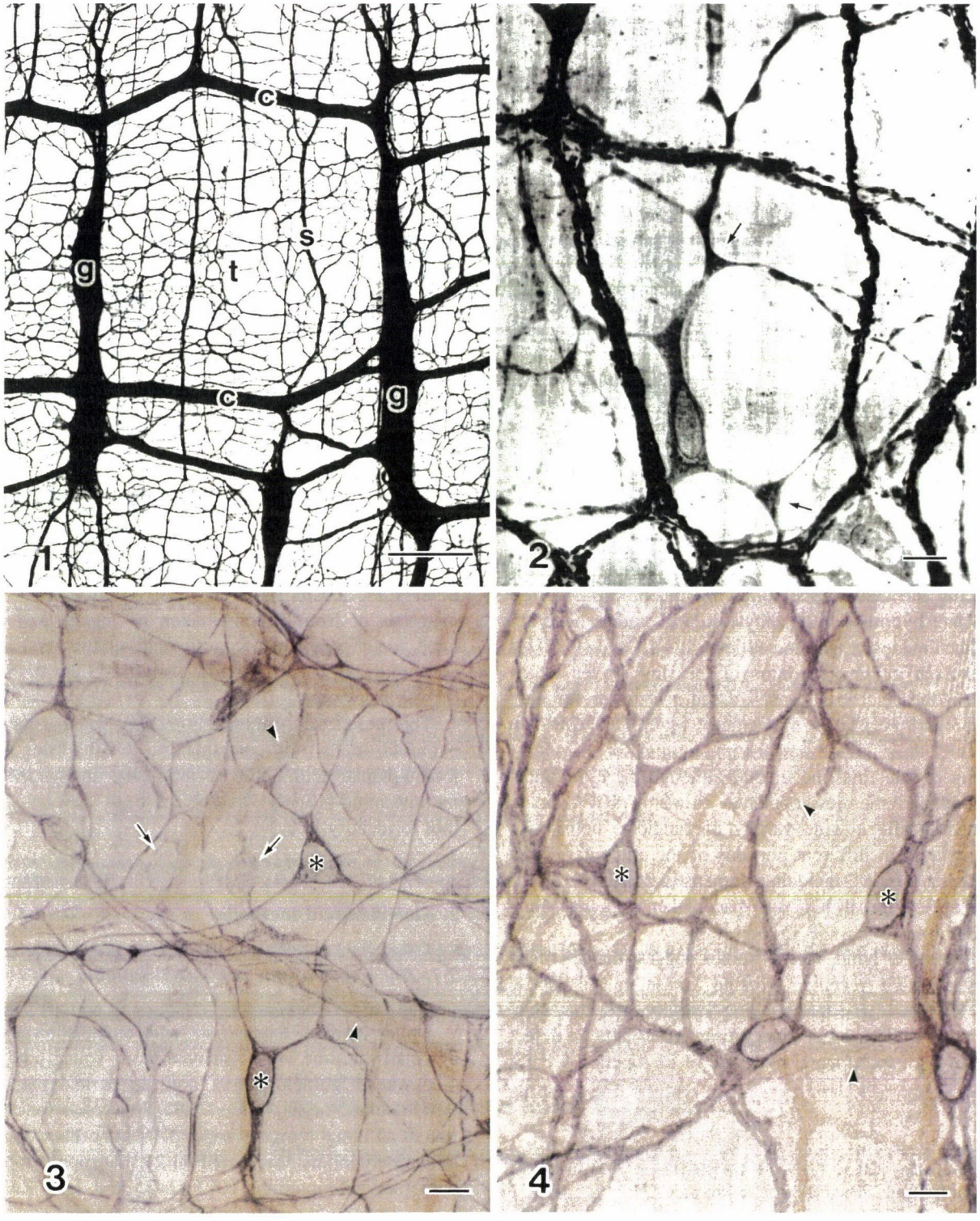
Zhou DS, Komuro T (1992b) The cellular network of interstitial cells associated with the deep muscular plexus of the guinea-pig small intestine. Anat Embryol 186:519–527

Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, Hsu, RY, Birkett NC, Okino KH, Murdock DC, Jacobson FW, Langley KE, Smith KA, Takeishi T, Cattanach BM, Galli SJ, Suggs SV (1990) Stem cell factor is encoded at the *Sl* locus of the mouse and is the ligand for the c-*kit* tyrosine kinase receptor. Cell 63:213–224

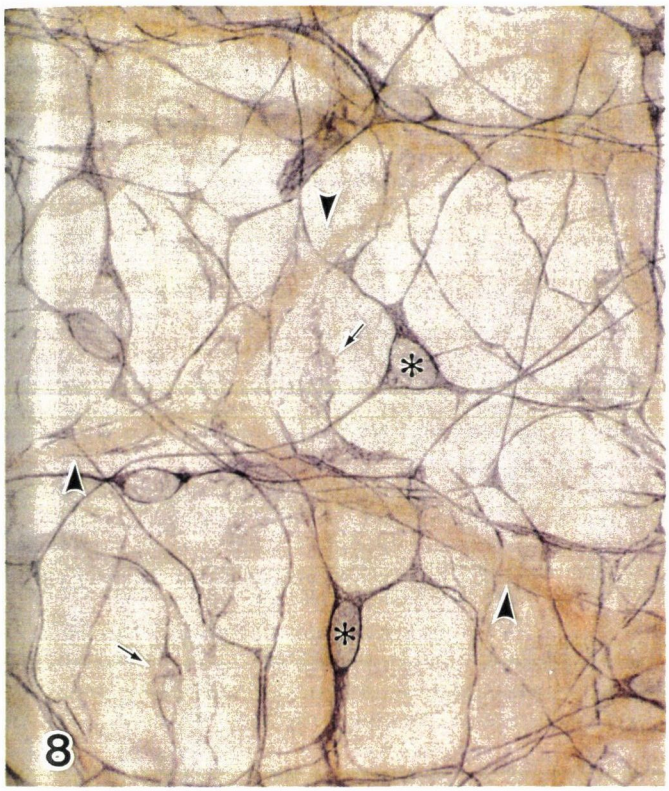
specimens were then rinsed in distilled water and cut along the mesentery to make flat sheets. Under a dissecting microscope, muscle layers with the myenteric plexus were carefully laminated with fine forceps. Those isolated pieces were distended and mounted with aqueous mounting medium for light microscopic examination.

2.2. Immunohistochemistry

Segments of guinea-pig small intestine were inflated and fixed for either 2 h at 4°C with fixative containing 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for vimentin, or 30 min at 4°C with absolute acetone for c-*kit*



located in meshes of the tertiary plexus and did not show close relations with nerve bundles. Three to five primary processes repeated the dichotomy to form secondary, tertiary and further branches. Therefore a single cell of this second type often spanned an area of several hundreds of microns in diameter. These processes connected with the same type of cells to form a distinct cellular network from the nerve plexus. However, in part, the processes appeared to make contacts with nerve bundles of the tertiary plexus. One of the most characteristic features of this cell type was the formation of a triangular knot at every branching point of the processes (Fig. 5). These cells were intensely stained with vimentin immunohistochemistry (Fig. 8). Cells



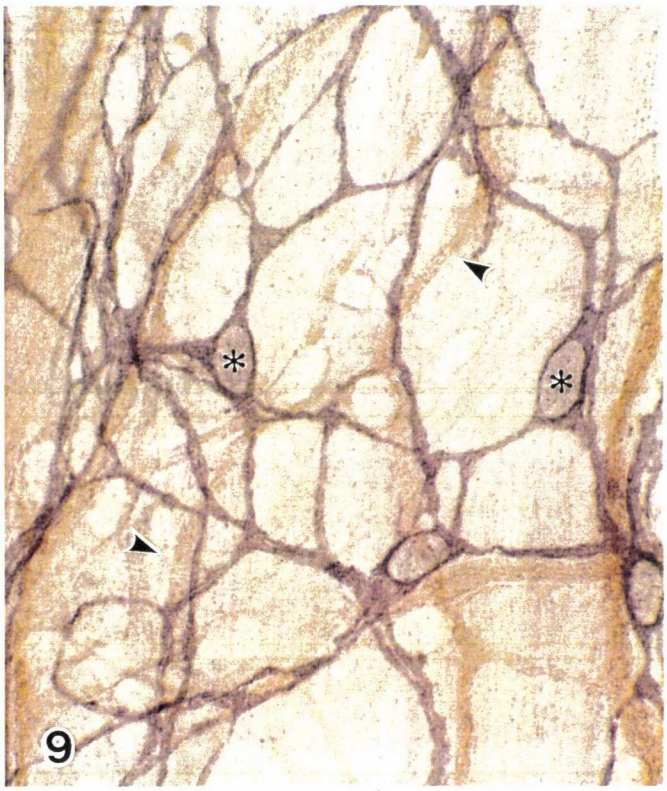


Fig. 8. The second type of myenteric interstitial cells revealed by immunohistochemical staining fro vimentin (*). Note the dichotomous branching pattern of the processes and the triangular knots at the branching points. Nerve bundles of the tertiary plexus are stained brown by the cholinesterase reaction (arrowheads). Independence of the cellular reticulum from the plexus is clear. Weakly-stained cells correspond to the first type of myenteric interstitial cells (arrows). From Komuro and Zhou, 1996. x 650

Fig. 9. Almost exactly the same shape of cells (*) as those in Fig. 8, demonstrated by immunohistochemical staining for c-kit receptor. The tertiary nerve plexus is stained brown by the cholinesterase reaction (arrowheads). From Komuro and Zhou, 1996. x 650

Fig. 1. Myenteric plexus of the guinea-pig small intestine-stained with ZIO method. The fine network of the tertiary plexus (t) is observed in the interstices of the primary plexus constituted by thick ganglion strands (g) and perpendicular connecting strands (c). Several nerve bundles of the secondary plexus (s), which directly connect the primary strands, are seen. ZIO staining. x 120

Fig. 2. A light micrograph showing interstitial cells (arrowheads) over the tertiary plexus and the cells of lymph vessels (arrows). ZIO staining. x 300

Fig. 3. Schwann cells located in the midst of the nerve bundles of the tertiary plexus stained with S-100 immunohistochemistry. x 700

Fig. 4. The first type of myenteric interstitial cells in the guinea-pig small intestine which is located beside the tertiary nerve. It extends several irregular processes. ZIO staining. x 850

Fig. 5. The second type of myenteric interstitial cell characterized by long cytoplasmic processes which show a dichotomous branching pattern. Note triangular knots at branching points (arrows). The cell body is usually seen apart from the nerve bundles. ZIO staining. x 700

Fig. 6. Stellate cells of the lymph vessel. ZIO staining. x 600

Fig. 7. A probable macrophage displaying a different appearance of inclusions revealed by ZIO staining. x 750