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Ultrastructural study of relationships between c-kit immunoreactive interstitial cells and other cellular elements in the human colon

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Abstract C-kit immunocytochemistry was performed on ultrathin sections of human distal colon. Our attention was focused on relationships between c-kit immunoreactive interstitial cells (c-kit ICs) and muscular cells and nervous elements located in the external muscular layers of the colonic wall. C-kit ICs established membrane apposition with both nerve fibers and smooth muscle cells of, respectively, the longitudinal and circular muscle layers, the myenteric area, and the extremus submucosus plexus. C-kit ICs also surrounded the external submucosus plexus and established membrane appositions with nerve elements located inside the myenteric ganglia. These membrane appositions were observed either at the level of the c-kit IC bodies or at that of their cytoplasmic processes. In some cases, membrane appositions were observed concomitantly between the c-kit ICs, nerve fibers, and smooth muscle cells. In all the regions studied, the c-kit ICs were also found to be located in the close vicinity of blood vessels and to have established close contacts with non-immunoreactive fibroblast-like cells. The results of the present study shed essential light on the relationships of c-kit ICs with the neighboring muscle cells and nerve elements, and confirm that the intercalated c-kit ICs well fit with the so-called "interstitial cells of Cajal".

Key words C-kit · Interstitial cells · Ultrastructure · Man · Colon

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Introduction

The knowledge available about the interstitial cells of Cajal (ICCs) in the mammalian digestive tract has progressed considerably over recent years, thanks to the discovery of the tyrosine kinase (c-kit) receptor (Yarden et al. 1987) they express. Two populations of morphologically distinct immunoreactive c-kit cells have been identified in the digestive tract of mammals and humans, namely the mastocytes (Vliagoftis et al. 1997) and the interstitial cells (Sanders 1996). C-kit immunoreactive interstitial cells (c-kit ICs) have been found to exist in various species, such as mice (Maeda et al. 1992; Huizinga et al. 1995; Torihashi et al. 1997; Ward et al. 1997), rats (Isozaki et al. 1995; Ishikawa et al. 1997; Horiguchi and Komuro 1998), and guinea pigs (Komuro and Zhou 1996; Burns et al. 1997; Seki et al. 1998), as well as humans (Horie et al. 1993; Matsuda et al. 1993; Rumessen 1994; Vanderwinden et al. 1996a,b; Hagger et al. 1997, 1998; Horisawa et al. 1998; Romert and Mikkelsen 1998; Torihashi et al. 1999; Wester et al. 1999). All in all, these studies have shown that the distribution and morphology of the c-kit ICs vary from one species to another, as well as from one region of the digestive tract to another, within a given species.

From the functional point of view, it has been suggested that the activation of the c-kit receptor by its ligand, the stem cell factor, may trigger the development of intestinal pacemaker activity (Maeda et al. 1992) as well as the contractile activity of muscle layers (Sato et al. 1996). The results obtained on *W* (Ward et al. 1994; Huizinga et al. 1995) and *Steel* mutant mice (Ward et al. 1995), or by injecting ACK2 antibodies (Tokutomi et al. 1995; Sato et al. 1996) have suggested that there may exist several different functional categories of c-kit ICs. The c-kit ICs located either in the myenteric area of the stomach and small intestine or in the extremus submucosus plexus of the colon may be involved in the genesis and propagation of slow waves (Sanders 1996) whereas those located in the myenteric region of the colon may be involved in the spatial coordination of motility and

the genesis of myenteric potential oscillations (Rae et al. 1998). Lastly, the c-kit ICs located inside the external smooth muscle layers of the stomach may participate preferentially in the regulation of neural inputs such as those mediated by NO (Burns et al. 1996). In humans, comparative studies on the distribution of the c-kit ICs between healthy and pathological tissue from patients with pyloric stenosis (Langer et al. 1995; Vanderwinden et al. 1996a), chronic idiopathic intestinal pseudo-obstruction (Isozaki et al. 1997), Hirschsprung's disease (Yamataka et al. 1995; Vanderwinden et al.1996b; Horisawa et al. 1998), and ulcerative colitis (Rumessen 1996) have shown that these diseases involve the disappearance of some populations of c-kit ICs, which argues in favor of the existence of several functional categories of c-kit ICs.

Almost all the data available in the literature, so far, on the c-kit ICs have been based on the results of light microscopic studies. In a recent transmission electron microscopic study on human colon, the ultrastructural characteristics of the c-kit ICs were defined (Torihashi et al. 1999). No ultrastructural data are available to date, however, on the relationships between the c-kit ICs and other cellular components of the digestive tract. The aim of the present study was therefore to investigate, in the human colon, these relationships at the ultrastructural level, focusing in particular on the cellular components involved in intestinal motility, namely the neuronal elements and the smooth muscle cells.

Materials and methods

Normal distal colon was obtained from four patients undergoing operative removal procedures for colonic carcinoma (one male and three females, aged 49–72 years, median 62 years). The patients had no other gastrointestinal diseases. After resection, the mucosa was gently excised and excluded from surgical specimens. Tissues were immediately fixed overnight in 4% paraformaldehyde solution in 0.1 M phosphate-buffered saline (PBS), pH 7.4 at 4°C, rinsed in PBS, cryopreserved overnight in sucrose (30%) at 4°C, embedded in Tissue Tek OCT compound (Miles, Elkhart, Ind., USA), and finally snap-frozen in CO_2. Tissues were cut on a cryostat into 30-µm-thick transverse sections. Free-floating sections were first incubated in 0.3% H_2O_2 solution in PBS, in order to inhibit endogenous peroxidase, for 20 min at room temperature. To minimize the non-specific labeling, sections were pretreated in 3% normal goat serum and 1% bovine serum albumin solution in PBS, for 1 h at room temperature. Tissues were then rinsed in PBS and incubated with rabbit polyclonal antibody against human c-kit receptor diluted at 1:200 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), for 24 h at 4°C. After being rinsed in PBS, the sections were incubated with a biotinylated goat anti-rabbit antibody (Jackson Laboratory, Bar Harbor, Me., USA) at a dilution of 1:200, for 1 h at room temperature, rinsed in PBS, and finally incubated with ABC coupled to horseradish peroxidase kit (Vector Laboratories, Burlingame, Calif., USA) diluted at 1:200, for 1 h at room temperature. The horseradish peroxidase reaction product was revealed by incubation with 0.04% 3,3′-diaminobenzidine (Sigma, St. Louis, Mo., USA), 2.5% nickel chloride (Sigma) and 0.01% H_2O_2 solution in 0.1 M sodium acetate buffer, for 5 min at room temperature. Sections were then immersed in 2% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for 45 min at room temperature. Finally, sections were dehydrated in a graded ethanol series and embedded in Durcupan (Fluka, Buchs, Switzerland). The specificity of the immunolabeling was checked by incubating the c-kit antiserum with its own antigen, SC-168p (Santa Cruz Biotechnology) at a concentration of 10 µM before incubating it with sections; this led to the complete absence of immunolabeling. No immunolabeling was also observed when the c-kit antiserum was omitted.

Semithin and ultrathin sections were cut using a Leica ultramicrotome. Semithin sections were mounted on slides and counterstained with azure blue. Sections showing relevant areas containing c-kit-immunopositive cells were selected and mounted on a Durcupan block and then cut out for ultrathin sections, which were mounted on formvar-coated grids, and contrasted with uranyl acetate and lead citrate before being examined with a Philips EM 400T electron microscope.

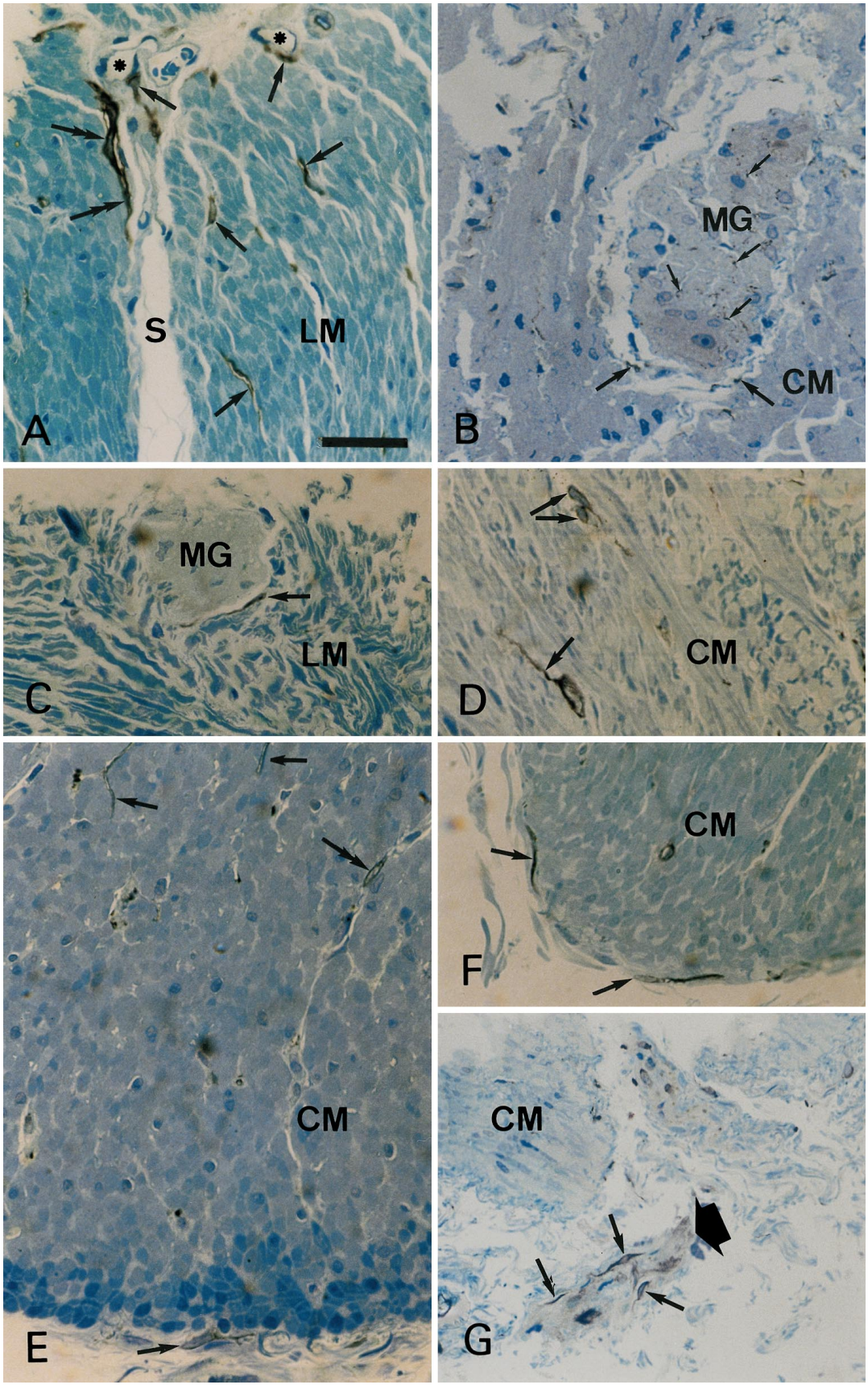
The present study was approved by the Ethics Committees of the Faculté de Médecine de Marseille (France).

Results

Immunohistochemistry

The c-kit ICs located in the longitudinal and circular muscle layers, the myenteric area containing the ganglionic and interganglionic regions, and at the interface between the inner circular muscle layer and the submucosa were characterized on semithin sections. In the longitudinal muscle layers, the c-kit ICs were found to be evenly distributed and to be oriented parallel to the longitudinal axis of the muscle fibers (Fig. 1A). They were characterized by long cytoplasmic processes inserted between the muscle cells. C-kit ICs were also present along the septa, where they were frequently interconnected, forming a dense network. In the myenteric area, c-kit ICs were detected around the myenteric ganglia (Fig. 1B,C) and in the interganglionic region. They contacted the ganglia and their processes were often wrapped around the ganglia (Fig. 1B,C) or even penetrated them (Fig. 1B). In the circular muscle layer, the distribution pattern of c-kit ICs was similar to that observed in the longitudinal muscle layer (Fig. 1D,E). At the interface between the inner circular muscle layer and the submucosa, c-kit ICs were dispersed over the extremus submucosus plexus within the connective tissue separating the inner circular muscle layer from the external submucosus plexuses (Fig. 1E,F) and around the external submucosus plexuses (Fig. 1G). In all the regions studied, the c-kit ICs were sometimes found to run beside blood vessels or to be wrapped around them (Fig. 1A).

Fig. 1A–G Semithin sections of human distal colon stained with azure blue. **A** Presence of c-kit interstitial cells (ICs) intercalated between muscle bundles (*arrows*) of the longitudinal muscle layer (*LM*), in the septa (*S, arrows with double heads*), and around blood vessels (*asterisks*). **B,C** C-kit ICs (*arrows*) surround a myenteric ganglion (*MG*) with, in **C**, a long and slender process; note in **B**, the presence of numerous c-kit-immunopositive processes (*small arrows*) inside the myenteric ganglion. **D,E** C-kit ICs (*arrows*) intercalated between muscle cells of the circular muscle layer (*CM*); note in **D**, the thin perinuclear cytoplasm and the long process and, in **E,** a c-kit IC in the septa (*arrows with double heads*). **E,F** In the extremus submucosus plexus, c-kit ICs (*small arrows*) are adjacent to the circular muscle cells. **G** C-kit ICs (*arrows*) surround an external submucous plexus (*thick arrow*). *Bar* 50 µm in **A,C–G**; 60 µm in **B** ▶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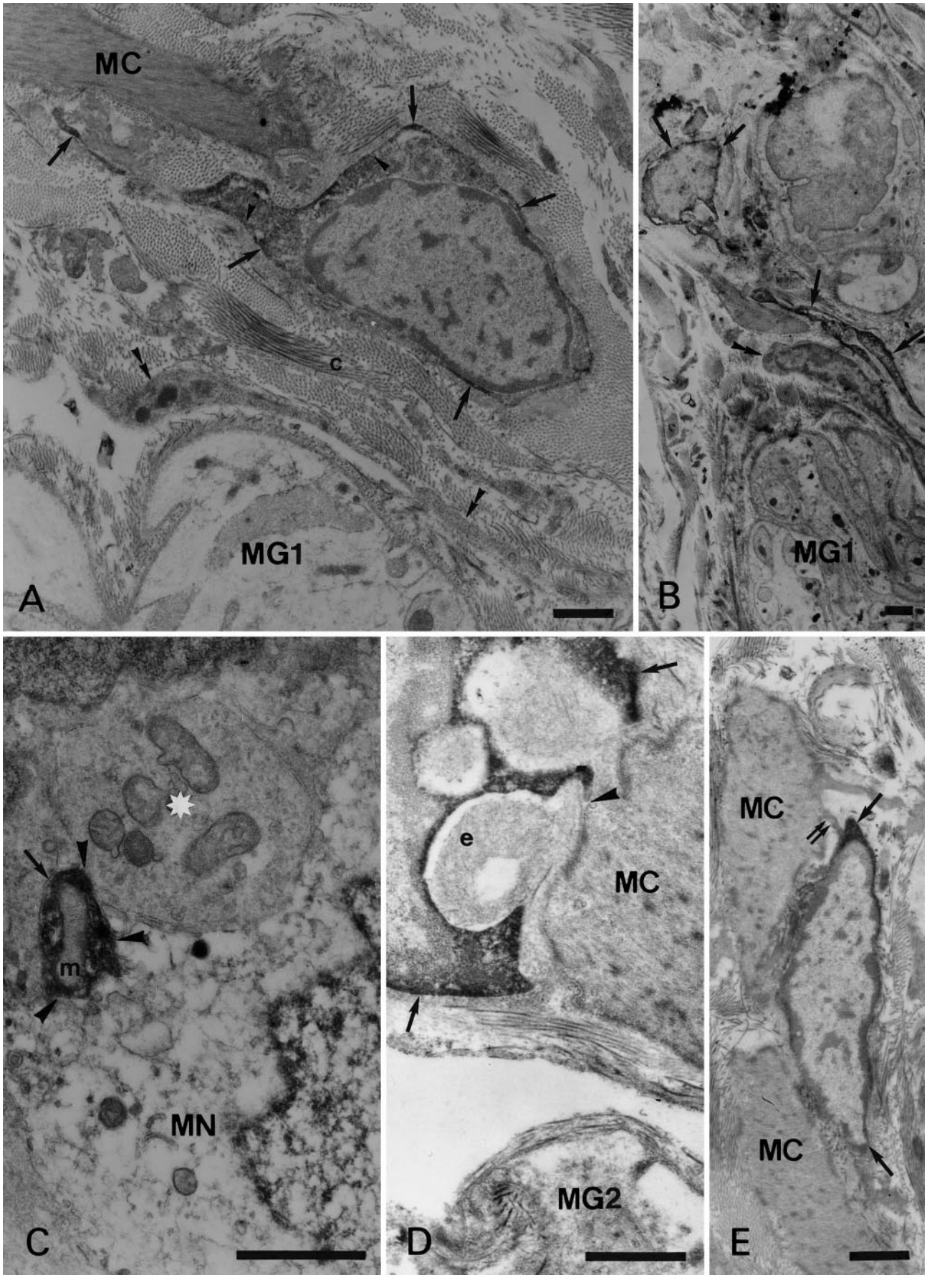
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Immunoelectron microscopy

In the longitudinal and circular muscle layers of the human colon, the c-kit ICs were the only c-kit immunoreactive cellular elements detected, apart from the mastocytes. All the other categories of cells observed, namely glial cells, Schwann cells, covering cells, and blood cells (polymorphonuclear granulocytes and macrophages in particular), were non-c-kit immunoreactive; a similar pattern of non-c-kit immunoreactivity emerged in the case of nerve fibers and muscle cells.

The c-kit ICs were characterized by a dense labeling which occurred along the plasma membrane. In some cases, this labeling was distributed in patches occurring along the plasma membrane (Fig. 2A), both at the level of the cell bodies and along the whole cytoplasmic processes. Dense c-kit reaction product was also diffusely scattered throughout the cytoplasm (Fig. 2D,E). The c-kit ICs were found to have a voluminous oval non-immunoreactive nucleus with a large amount of marginal heterochromatin and widely dispersed chromatin (Fig. 2A,E). The c-kit ICs had a narrow perinuclear cytoplasm (Figs. 2A,B,E, 5A) and long cytoplasmic processes which projected for long distances between the surrounding cells (Fig. 2B). Their cytoplasm contained numerous mitochondria and caveolae (Figs. 2A,C, 5A), a few secondary lysosomes, microfilaments, and intermediate filaments (Fig. 4B). The c-kit ICs had a discontinuous basal lamina and invaginations containing aggregates of elastin (Figs. 2D, 3D). In some cases, the c-kit ICs located in the connective tissue, interposed between the extremus submucosus and the external submucosus plexuses, showed fibrils of collagen which contacted their plasma membrane (Fig. 4D).

In all three regions studied, i.e., the myenteric area, the external muscle layer, and the interface between the inner circular muscle layer and the submucosa, the c-kit ICs established relationships with cells of various kinds. These relationships could be either membrane appositions, in which case the intercellular space was less than 50 nm in size, or close proximity (up to 2 µm). In the present study, we focused in particular on the membrane appositions occurring between c-kit ICs on the one hand and smooth muscle cells and nerves on the other. The density of the immunoreactive labeling observed in c-kit ICs did not allow us to identify clearly the presence of any membrane thickening at the level of these cells, which made it impossible to determine the exact nature of the membrane appositions observed. The membrane appositions, along with the respective nerves and smooth muscle cells, could be located either at the level of the c-kit IC bodies or at that of their cytoplasmic processes.

It is worth recalling here that, in humans, there exists an anatomical specificity as far as the myenteric ganglia are concerned (Faussone-Pellegrini et al. 1990b). These ganglia, which can be deeply buried in either the longitudinal or circular muscle layer, are of two kinds: the type 1 ganglia (MG1), which are surrounded by a single sheath of connective tissue, and sometimes also by an additional discontinuous layer of covering cells (Fig. 2A), and the type 2 ganglia (MG2), which are surrounded by a further muscle sheath consisting of two to ten layers of muscle cells (Fig. 3C). At the level of the MG1 ganglia, the cytoplasmic processes of c-kit ICs were wrapped closely around the ganglia (Fig. 2A,B); the processes sometimes even penetrated the ganglia and formed membrane appositions with the soma of myenteric neurons and nerve varicosities (Fig. 2C). As far as the MG2 ganglia were concerned, c-kit ICs were detected in the enveloping muscle sheath (Fig. 2D,E). Here, they formed membrane appositions with the muscle cells comprising the sheath; the basal lamina of the muscle cells sometimes extended as far as the plasma membrane of the c-kit ICs (Fig. 2D). The c-kit ICs were also sometimes linked to the smooth muscle cells by elastin bridges (Fig. 2E). In the interganglionic region, the c-kit ICs established membrane appositions with the nerve fibers and the muscle cells.

Inside the longitudinal and circular muscle layers, the c-kit ICs established membrane appositions with nerve bundles (Fig. 3A,B) and muscle cells (Fig. 3B–D); appositions of this kind were also observed within the septa. In some cases, we observed contacts occurring concomitantly between the c-kit ICs, nerve fibers, and muscle cells (Fig. 3B). At the interface between the inner circular muscle layer and the submucosa, c-kit ICs belonging to the extremus submucosus plexus were observed in the proximity of muscle cells and nerves (Fig. 4A,B); in this

◀ **Fig. 2A–E** Electron micrographs of c-kit ICs in relation to myenteric ganglia either devoid (*MG1*) or surrounded by a myenteric muscle sheath (*MG2*). **A,B** C-kit ICs (*arrows*) in close proximity of an MG1; c-kit ICs are coated with collagen (*c*) and separated from the ganglion by unlabeled covering cells (*double arrowheads*); in **A**, c-kit IC process is in close proximity with a longitudinal muscle cell (*MC*); note also the presence in the plasma membrane of caveolae (*small arrowheads*). **C** C-kit IC process (*arrow*) in an MG1 containing a mitochondria (*m*), which is simultaneously apposed to a myenteric neuron (*MN*, *arrowheads*) and a varicose nerve (*white asterisk*) containing numerous mitochondria. **D,E** Interrelationships between c-kit ICs (*arrows*) and muscle cells (*MC*) of the muscle sheaths surrounding an *MG2*; in **E**, c-kit IC is linked to the muscle cell by an elastin bridge (*double arrows*). *e* Elastin. *Bars* 1 µm

Fig. 3A–D Electron micrographs of c-kit ICs closely apposed to nerve and muscle cells in the circular and longitudinal muscle layers. **A,B** In septa, c-kit IC processes (*arrows with double heads*) in close association with nerve fiber bundles (*NFB*). **A** C-kit IC process apposed (*arrowhead*) to a nerve fiber containing large granular vesicles. **B** Long c-kit IC process containing mitochondria simultaneously apposed to a nerve bundle and a muscle cell (*MC*). **C** C-kit IC (*arrows*) apposed to a longitudinal muscle cell in the vicinity of *MG2* enveloped by a myenteric muscle sheath (*MMS*). **D** High magnification of a portion of **C** (*large arrow*); note the close apposition (*arrowhead*) between c-kit IC (*arrows*) and a muscle cell; numerous elastin bundles (*e*) are present within invaginations of a c-kit IC process. *c* Collagen. *Bars* 1 µm ▶

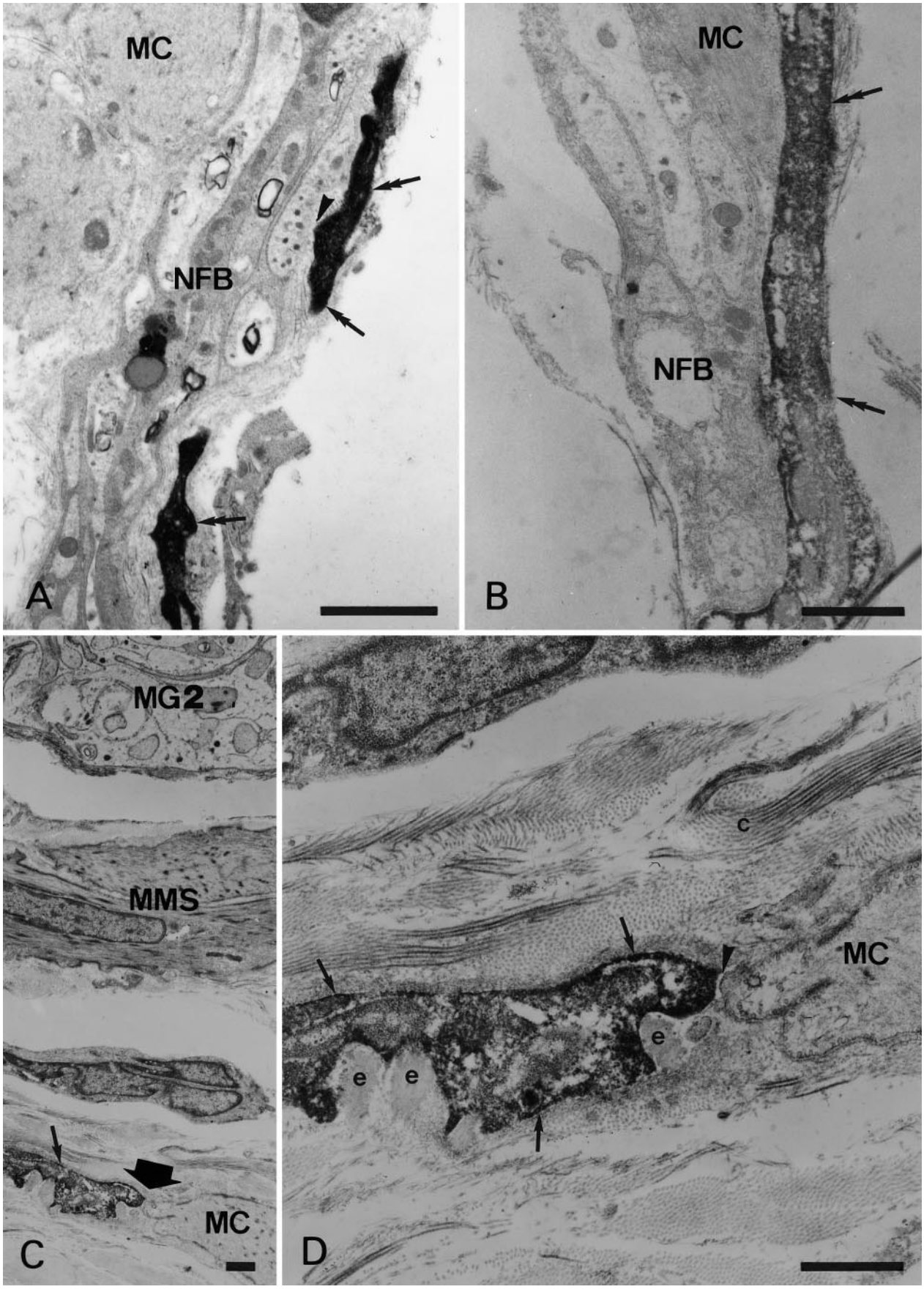

Fig. 3A–D

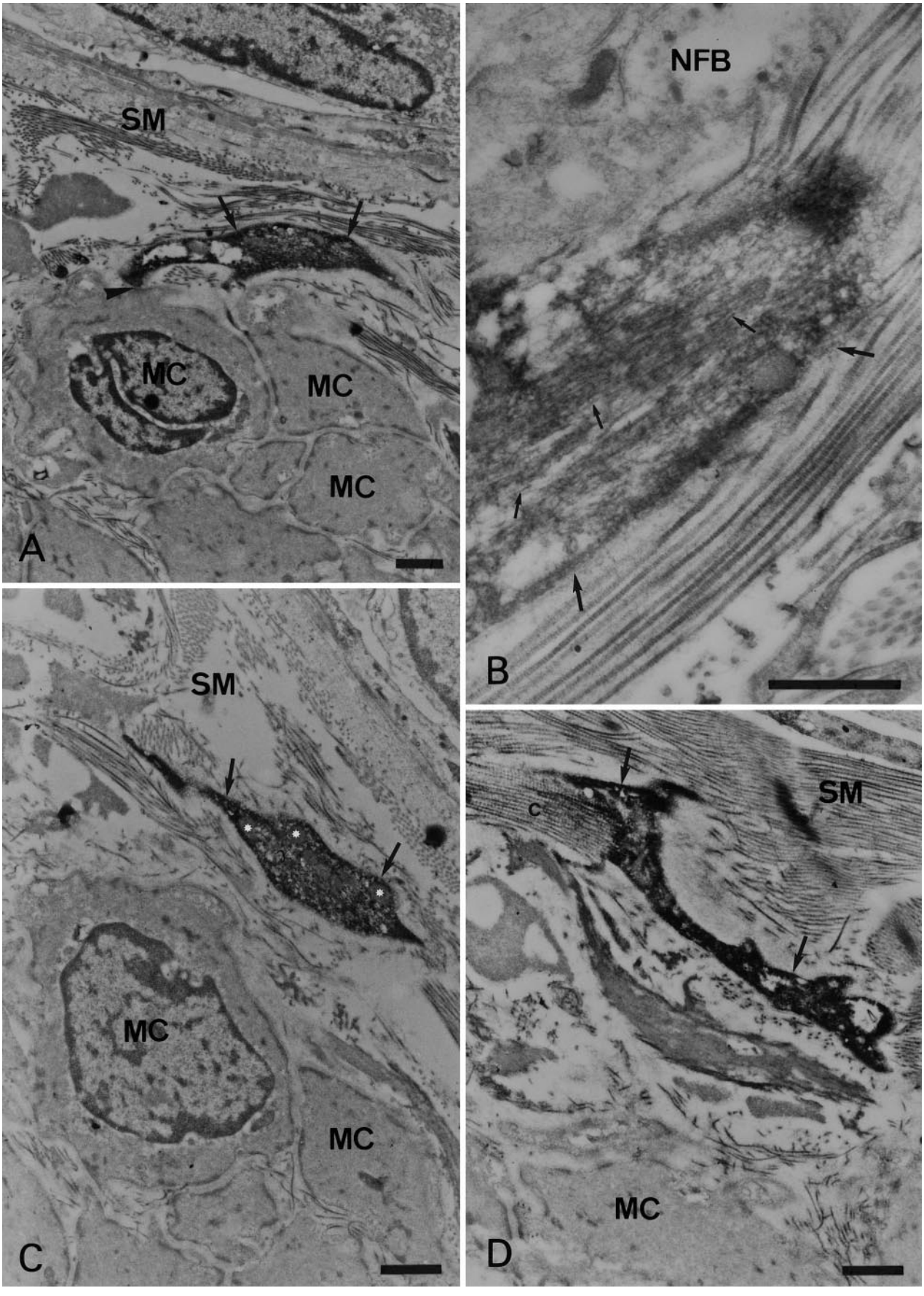

Fig. 4A–D

case, membrane appositions were observed with the muscle cells (Fig. 4A). In the connective tissue, bundles of collagen fibers established contacts with the plasma membrane of the c-kit ICs (Fig. 4D).

In all the regions studied, the c-kit ICs were also found to be located in the close vicinity of blood vessels (Fig. 5A,C) and to have established close contacts with non-immunoreactive fibroblast-like cells (Fig. 5B).

Discussion

The results of the present study indicates that c-kit ICs are present in all the muscular layers of the distal colonic wall studied here, in agreement with the previously published immunohistological (Horie et al. 1993; Matsuda et al. 1993; Yamataka et al. 1995; Rumessen 1996; Vanderwinden et al 1996b; Hagger et al. 1997; Romert and Mikkelsen 1998) and immunocytological (Torihashi et al. 1999) data.

The present results show that the c-kit ICs in the external muscle layers are of the myoid type which is consistent with other recently published data (Torihashi et al. 1999). The ultrastructural features of the c-kit ICs characterized in this previous study and the present one were found to be similar to those of the ICCs located in the same regions of the colonic wall (Faussone-Pellegrini et al. 1990a,b; Rumessen et al. 1993; Rumessen 1994). It therefore seems very likely that the c-kit ICs of the myoid type are ICCs expressing the c-kit receptor. The possibility cannot however be ruled out that there may exist ICCs which do not express the c-kit receptor (Torihashi et al. 1999), or which do so only under specific conditions, as found to occur in the case of the ICCs present in the deep muscular plexus of the mouse (Wester et al. 1999). Another category of c-kit ICs, including ICs of a mixed type, was found here to exist at various levels in the colonic wall, especially at the interface between the inner circular muscle layer and the submucosa. These c-kit ICs were not involved in any very close relationships with the surrounding nerve fibers and muscle cells. They might be taken as fibroblast cells which have become differentiated, depending on their relationships with the surrounding cellular microenvironment (Komuro 1990).

Although previous authors have concluded that there exist no ICCs in the longitudinal and circular muscle layers of the human colon (Christensen and Rick 1987; Faussone-Pellegrini 1987; Rumessen 1994), the present data show the existence of c-kit ICs in both of these muscle layers as well as in the septa, in line with other previous studies (Hagger et al. 1997; Torihashi et al. 1999). Here we have shown, in addition, that c-kit ICs are frequently inserted between the nerve fibers and the muscle cells, with which they establish membrane appositions. This latter finding indicates that these c-kit ICs correspond to the ICCs defined as being non-nerve and non-muscle cells interposed between nerve fibers and smooth muscle cells (Thuneberg 1989). Their close relationships with nerve and muscle elements strongly suggest that they may be directly involved in the neuroregulatory processes underlying colonic motility (Hagger et al. 1997). It is worth mentioning that recent electrophysiological findings have suggested that the ICCs located in the median part of the colonic circular muscle layer may also be involved in the rhythmic motor activity (Rae et al. 1998).

The presence of c-kit IC processes inside the myenteric ganglia of the human colon has never been clearly established so far on the basis of immunohistological data. Up to now, either their existence has been completely ruled out (Romert and Mikkelsen 1998) or c-kit immunoreactivity has been reported to occur only in some of the glial cells (Matsuda et al. 1993). The results of the present study show that the expression of the c-kit receptor takes place only on the c-kit ICs closely surrounding and sometimes penetrating the myenteric ganglia, MG1, devoid of a muscle sheath. These c-kit ICs are probably identical to the ICCs previously described in the myenteric plexuses of the human colon (Faussone-Pellegrini et al. 1990b). The exact function of the ICCs surrounding the myenteric plexuses has not yet been determined. The present finding that there exist close relationships between processes projecting from the c-kit ICs and the ganglionic neurons certainly suggests that the c-kit ICs may be involved in ganglionic neurotransmission. In addition, recently published electrophysiological data have suggested that they may participate in the genesis of the myenteric potential oscillations occurring in the smooth muscle cells in the longitudinal and circular layers (Rae et al. 1998). As far as the c-kit ICs located at the interface between the longitudinal and circular muscle layers are concerned, if one makes analogies with the ICCs located in the same region, it seems likely that they may be involved in particular in the electrical coupling between the longitudinal and circular muscle layers, which is responsible for the coordination of motor activities (Rae et al. 1998).

In the present study, c-kit ICs belonging to the plexus entericus extremus located at the interface between the inner circular muscle layer and the submucosa were found to be present. This result is in agreement with previous data obtained in other mammals (Stach 1972; Berezin et al. 1988). Although this finding is not in agreement with the data obtained by some previous authors, who observed the presence of these cells only at the level of the proximal colon (Romert and Mikkelsen 1998), it corroborates that of other authors, who detected ICCs at the level of the distal colon (Vanderwinden et al.

◀ **Fig. 4A–D** Electron micrographs of c-kit ICs located in the submucosa. **A** C-kit IC process (*arrows*) closely apposed (*arrowhead*) to a muscle cell (*MC*). **B** High magnification of a c-kit IC process (*arrows*) containing numerous intermediate filaments (*small arrows*) in close proximity of a nerve fiber bundle (*NFB*). **C,D** C-kit IC processes (*arrows*) in the vicinity of muscle cells (*MC*); note the presence in **C** of numerous mitochondria (*white asterisks*) and, in **D**, collagen bundles (**c**) closely associated with the c-kit IC. *SM* Submucosa. *Bars* 1 µm

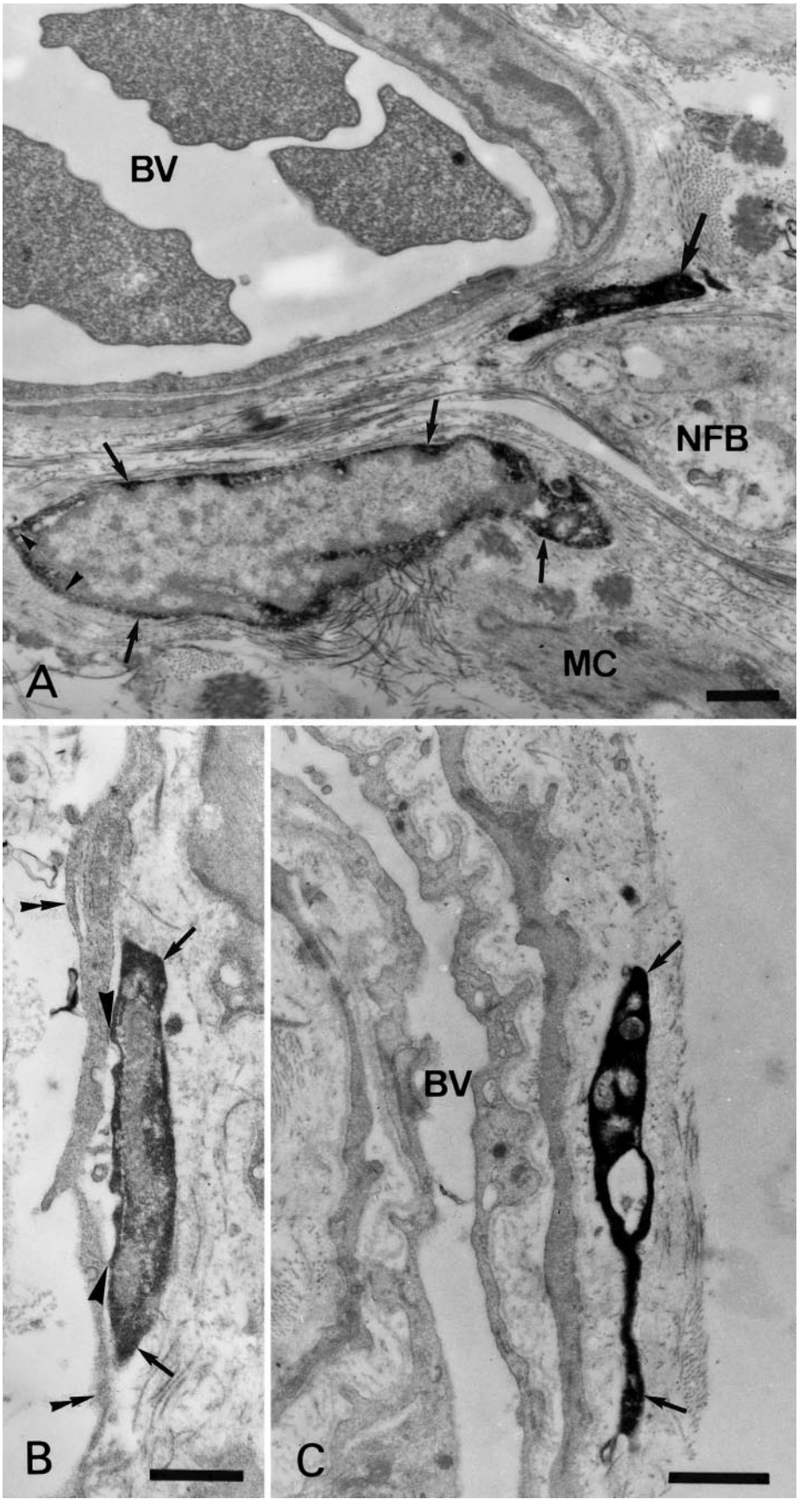

Fig. 5A–C Electron micrographs of interrelationships between c-kit ICs, blood vessels, and a fibroblast-like cell. **A** In the myenteric area, a c-kit IC process (*large arrow*) in close proximity of a blood vessel (*BV*); note in the vicinity of the c-kit IC (*small arrows*) containing caveolae (*small arrowheads*), the presence of a nerve fiber bundle (*NFB*) and a muscle cell (*MC*). **B,C** In the circular muscle layer, c-kit ICs (*arrows*) are, in **B**, closely apposed (*arrowheads*) to an unlabeled fibroblast-like cell (*double arrowheads*) and, in **C**, run in the close proximity of a blood vessel (*BV*). Bars 1 µm

1996b; Hagger et al. 1997; Torihashi et al. 1999). We established here that c-kit ICs belonging to the plexus entericus extremus frequently establish relationships with the inner circular muscle cells. The existence of these relationships supports the idea that the ICCs of the plexus entericus extremus may be involved in the initiation of the slow peristaltic waves in the circular muscle layer, in line with recent electrophysiological data (Rae et al. 1998).

In conclusion, the results of the present study shed essential light on the ultrastructural characteristics of the c-kit ICs, especially as regards their relationships with the neighboring muscle cell and nerve elements. Altogether, these data unequivocally confirm the idea that the expression of the c-kit receptors takes place at the level of most ICCs. The role played by the c-kit receptors along with the ICCs and their ligand, the stem cell factor, still remains to be elucidated. Further studies are therefore now required on this topic, in order to improve our knowledge of the regulatory mechanisms underlying intestinal motility.

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