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An Ultrastructural Study of Cell-Cell Contact between Mouse Spleen Cells and Calvaria-Derived Osteoblastic Cells in a Co-Culture System for Osteoclast Formation

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We have previously established a mouse co-culture system of osteoblastic cells and spleen cells for examining osteoclast differentiation. In the present study, we examined the morphological features of the cell-cell contact between mouse spleen cells and osteoblastic cells in the co-cultures. Light microscopic investigations revealed that tartrate-resistant acid phosphatase (TRAP)-positive mononuclear and multinucleated spleen cells appeared in the vicinity of alkaline phosphatase (ALP)-positive osteoblastic cells. Ultrastructurally, spleen cells extended long cytoplasmic filopodia in all directions, by which spleen cells touched adjacent osteoblastic cells. The adjacent osteoblastic cells and spleen cells adhered to each other by forming electron-dense cytoplasmic materials on their inner leaflets of plasma membranes at cell-cell contact sites. Some adjacent osteoblastic and spleen cells made contact on the plasma membranes, forming an extracellular microenvironment. Coated pits were also formed on the plasma membranes facing this microenvironment. These morphological features of the cell-cell contact between osteoblastic cells and spleen cells indicate that there is internalization of organic components, i.e., receptor-mediated endocytosis in the contact sites between the two types of cells.

Key words: Co-culture, Osteoclast formation, Osteoblastic cells, Cell-cell contact, Microenvironment

I. Introduction

It is well established that osteoblastic stromal cells are essential for osteoclast differentiation. We have previously established a mouse co-culture system of osteoblastic cells and spleen cells for examining osteoclast differentiation [27-29]. Multinucleated cells formed in this co-culture system in response to bone-resorbing factors such as 1α, 25-dihydoxyvitamin D_3 [1α, $25(OH)_2D_3$], parathyroid hormone (PTH), prostaglandin E_2 (PGE_2) and interleukin 1 (IL-1) showed not only tartrate-resistant acid phosphatase (TRAP) activity and abundant calcitonin receptors [4, 30], but also resorbed bone and dentine with typical ruffled borders [22]. The characteristics of these multinucleated cells generated in this co-culture system, therefore, satisfied the major criteria for authentic osteoclasts [1, 10, 17, 18]. Cell-cell contact between spleen cells and osteoblastic stromal cells is thought to be necessary for the osteoclast formation, since no TRAP-positive osteoclasts were formed when the two types of cells were co-cultured without direct contact even in the presence of bone-resorbing factors.

In the present study, therefore, we examined the morphological features of the cell-cell contact between mouse calvaria-derived osteoblastic cells and spleen cells in co-cultures. Spleen cells extended long cytoplasmic filopodia in all directions, by which spleen cells touched adjacent osteoblastic cells. The two types of cells adhered to each other by forming electron-dense cytoplasmic materials on their inner leaflets of the plasma membranes at the contact sites, suggesting that there is receptor-mediated endocytosis in the contact sites between osteoblastic cells and spleen cells.

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II. Materials and Methods

Animals and Chemicals

Pregnant mice and 6- to 8-week old male mice, both ddY strain, were purchased from Nihon SLC, Co. (Hamamatsu, Japan). 1α, 25$(OH)_2D_3$ was kindly provided by Chugai Pharmaceutical Co. (Tokyo, Japan) and [^{125}I]-labeled and unlabeled eel calcitonin (elcatonin) from Asahi Chemical Industry (Tokyo, Japan). Collagenase and dispase were purchased from Wako Pure Chemicals (Osaka, Japan) and Sankyo Pure Chemicals (Tokyo, Japan), respectively.

Co-culture system

Osteoblastic cells and spleen cells were prepared as previously described [27]. In brief, calvariae were peeled out of fibrous layers and blood vessels, and immediately incubated in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% collagenase and 0.2% dispase at 37°C. Osteoblastic cells showing intense ALPase activity were obtained by such collagenase digestion. Spleen cells were isolated by grating splenic tissues of 6- to 8-week old ddY mice on metal meshes. Before co-culturing, either round (ϕ13 mm) glass coverslips (Matsunami, Co., Tokyo, Japan) or plastic coverslips (Termanox, Nunc, Inc., Naperville, IL) were placed on 24-well culture plates (Corning, Corning, NY) to allow for subsequent light and electron-microscopic observations, respectively. Osteoblastic cells (1×10^4/well) and spleen cells (5×10^5/well) were co-cultured in the 24-well plates with 1ml of α-minimum essential medium (α-MEM, Flow Laboratories, Irvine, Scotland) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Cosmo Bio. Co. Ltd., Tokyo, Japan). Co-cultures were performed either in the presence or absence of 10^{-8} M 1α, 25$(OH)_2D_3$ for 4 or 7 days. During that time, all co-cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, replacing 0.5 ml old medium with fresh medium every 3 days.

Double staining for TRAP and ALP activities

The cells co-cultured for 4 or 7 days, either in the presence or absence of 1α, 25$(OH)_2D_3$, were fixed with 4% paraformaldehyde in 0.067 M cacodylate buffer (pH 7.4) for 1 hr. TRAP and ALP activities were detected simultaneously on all sections by using Burstone's Azo dye method [3]. In this procedure, ALP staining was carried out prior to the TRAP detection. Specimens were incubated in a mixture of 9 mg of naphtol AS-BI phosphate (Sigma, St. Louis, MO, USA) and 54 mg of fast blue LL salt (Sigma) diluted in 100 ml of 0.1 M Tris-HCl buffer (pH 8.5). For TRAP staining, these specimens were rinsed with PBS and incubated in a mixture of 8 mg of naphtol AS-BI phosphate, 70 mg of red violet LB salt (Sigma) and 50 mM L(+) tartaric acid (0.76 g, Nacalai Tesque, Tokyo, Japan) diluted in 0.1 M sodium acetate buffer (pH 5.0). Each incubation was performed for 15 min at 37°C.

Autoradiography with [^{125}I]-Elcatonin

The cells co-cultured for 7 days, in the presence of 1α, 25$(OH)_2D_3$ were incubated with α-MEM supplemented with 0.1% bovine serum albumin and 14.2 nM [^{125}I]-elcatonin (2.5 μCi/ml) for 15 min. After rinsing with cold α-MEM, co-cultures were fixed with 4% paraformaldehyde in 0.067 M cacodylate buffer (pH 7.4), for 1 hr, then examined for TRAP activity. The specimens were dehydrated in increasing concentrations of ethanol, air-dried and dipped with Konica NR-M2 emulsion (Konishiroku Photo Industry, Tokyo, Japan). They were exposed for 2 weeks at 4°C in a dark room, developed with Konicadol X (Konishiroku) for 4 min at 20°C and fixed with Super Fuji Fix (Fuji Photo Film Co., Tokyo, Japan). The specificity for [^{125}I]-elcatonin binding was further assessed by incubation with the mixture of 14.2 nM [^{125}I]-elcatonin and an excess amount (300 nM) of unlabeled elcatonin.

Ultrastructural observation

For scanning electron microscope (SEM) studies, osteoblastic cells and spleen cells co-cultured in the presence of 1α, 25$(OH)_2D_3$ for 7 days were fixed using a mixture of 1% paraformaldehyde and 2% glutaraldehyde in 0.067 M cacodylate buffer (pH 7.4) for 4 hr at 4°C. The specimens were rinsed with 0.1 M of the same buffer (pH 7.4), and postfixed according to the OsO_4-tannic acid-OsO_4 method [2]. They were then dehydrated using an ever-increasing concentration of acetone and critical-point dried and spatter-coated with gold for SEM observation (Hitachi S-570, Hitachi Co., Ltd., Tokyo, Japan).

Transmission electron microscope (TEM) studies employed 7-day old co-cultured cells in the presence of 1α, 25$(OH)_2D_3$. They were fixed with 4% glutaraldehyde in 0.067 M cacodylate buffer (pH 7.4) for 4 hr at 4°C. Specimens were dehydrated in increasing concentrations of ethanol and embedded in Epok 812 (Ohken Shoji Co., Tokyo, Japan). Ultrathin cross-sections of the co-cultures were stained with uranyl acetate and lead citrate for 2–5 min prior to TEM observation (Hitachi H-500, Hitachi Co.).

Immunocytochemistry for F4/80

The cells, which were co-cultured for 4 or 7 days in the presence of 1α, 25$(OH)_2D_3$ were fixed with 4% paraformaldehyde in 0.067 M cacodylate buffer for 1 hr. The specimens were preincubated with 1% bovine serum albumin in phosphate buffered saline (BSA-PBS) for 30 min, and then incubated with culture supernatant of hybridoma cells secreting F4/80 as described previously [19]. The specimens were incubated with FITC-conjugated goat anti-rat Igs at a dilution of 1 : 100 (Kirkegaard & Perry laboratories Inc. Gaithersburg, MD, USA) The cultured cells were observed under fluorescent microscpy.

III. Results

Light microscopic observations

Figure 1 shows localization of TRAP-positive mononuclear and multinucleated cells formed in the co-cultures in the presence or absence of 1α, $25(OH)_2D_3$ on days 4 and 7. On day 4, neither TRAP-positive mononuclear nor multinucleated cells were formed in the absence of 1α, $25(OH)_2D_3$ (Fig. 1A). Osteoblastic cells were positively stained for ALP in the absence of the vitamin (Fig. 1A). In the presence of 1α, $25(OH)_2D_3$, however, many TRAP-positive spleen cells appeared on day 4 (Fig. 1B). They were seen in contact with ALP-positive cells. These TRAP-positive spleen cells were round in shape, and mononuclear on day 4. Most of the spleen cells located apart from the ALP-positive cell were TRAP-negative (Fig. 1B). When co-cultures were performed for 7 days without 1α, $25(OH)_2D_3$, a small number of TRAP-positive spleen cells were observed, most of which were mononuclear (Fig. 1C). When cells were co-cultured with 1α, $25(OH)_2D_3$ for 7 days, numerous TRAP-positive mononuclear and multinucleated cells appeared in the vicinity of ALP-positive osteoblastic cells (Fig. 1D).

Light microscopic autoradiography demonstrated that many silver grains of [^{125}I]-elcatonin were present over TRAP-positive multinucleated cells (Fig. 1E). No labeling was seen over the TRAP-negative spleen cells and osteoblastic cells. The labeling was markedly reduced when an excess amount of unlabeled elcatonin was added (data not shown).

Immunofluorescent examination revealed many F4/80-immunopositive cells in the co-cluture on both 4 and 7 days (Fig. 2). F4/80 recognizes murine macrophage-restricted cell surface glycoproteins [12, 13, 19]. In our own observation, F4/80-immunopositive cells were not only observed when osteoblastic cells were cultured, which led us to conclude that F4/80 can recognize spleen-derived macrophage-monocyte lineages. On day 4, F4/80-immunoreactivity was observed on the cell surfaces of round spleen cells (Fig. 2A). On day 7, some spreading cells lost F4/80-immuopositivity on their cell surfaces, although round F4/80-immunopositive spleen cells were present in abundance (Fig. 2B).

SEM observations

In the co-cultures incubated with 1α, $25(OH)_2D_3$ for 7 days, a number of spherical spleen cells formed clumps on flattened osteoblastic cells which were spread over on the plastic dish (Fig. 3A). These spleen cells were characterized by many fine cytoplasmic processes and/or membrane-foldings. Spleen cells occasionally extended long filopodia in all directions, making contact with neighbouring osteoblastic cells (Fig. 3B). Cytoplasmic processes of surrounding osteoblastic cells were also observed, which made contact with those of spleen cells. Neighbouring spherical spleen cells also made contact with each other by means of numerous fine cytoplasmic processes (Fig. 3C). Giant, dome-like spleen cells were also seen in close contact with osteoblastic cells (Fig. 3D).

TEM observations

Figure 4 shows TEM observations of cross-sectioned co-cultures at a low magnification. Mononuclear spleen cells with rudimentary cell organelles were located on the flattened osteoblastic cells (Fig. 4A). Some of these spleen cells were anchored to osteoblastic cells by short cytoplasmic processes. Other spleen cells having numerous mitochondria and vacuoles were observed to creep underneath osteoblastic cells. Multinucleated spleen cells extended cytoplasmic projections underneath osteoblastic cells as well (Fig. 4B). Like an authentic osteoclast, these multinucleated cells showed abundant mitochondria scattered throughout the cytoplasm (Fig. 4C).

Ultrastructures of the cell-cell contact between osteoblastic cells and spleen cells at a high magnification are shown in Fig. 5. As has been shown in Fig. 4A, clumps of spleen cells adhered to osteoblastic cells. Electron-dense materials were observed on the inner leaflets of the plasma membranes at the contact sites (Fig. 5A), presumably being identical to adherens junctions. Thin extracellular matrix was often observed between the cell membranes of spleen cells and osteoblastic cells, thus, indicating that osteoblastic cells and spleen cells freely interact via thin extracellular matrix and direct cell-cell contacts.

Adjacent osteoblastic cells and spleen cells often formed contact on the plasma membranes, which resulted in the formation of a narrow extracellular space between the cells (Fig. 5B). The narrow extracellular space formed was sealed with contact, resulting in an intercellular microenvironment. In this microenvironment, the plasma membranes

Fig. 1. Localization of TRAP-positive mononuclear and multinucleated cells formed in co-cultures on days 4 and 7 in the presence and absence of 1α, $25(OH)_2D_3$. TRAP-positive cells are shown in red and ALP-positive cells in blue. **(A)** On day 4, only ALP-positive cells are seen in co-cultures in the absence of 1α, $25(OH)_2D_3$. Original magnification = ×400. Bar = 25 μm. **(B)** In the presence of 1α, $25(OH)_2D_3$, TRAP-positive mononuclear cells appear in contact with ALP-positive cells on day 4 (arrows). Spleen cells located apart from ALP-positive cells were negative for TRAP-activity. Original magnification = ×400. Bar = 25 μm. **(C)** When cells were co-cultured without 1α, $25(OH)_2D_3$ for 7 days, a limited number of TRAP-positive mononuclear cells appeared in the vicinity of ALP-positive cells. Original magnification = ×400. Bar = 25 μm. **(D)** In the presence of 1α, $25(OH)_2D_3$, many TRAP-positive mononuclear and multinucleated cells are formed. Original magnification = ×400. Bar = 25 μm. **(E)** Autoradiography of [^{125}I]-elcatonin binding. Numerous silver grains are seen over TRAP-positive mononuclear and multinucleated cells. Original magnification = ×800. Bar = 12 μm.

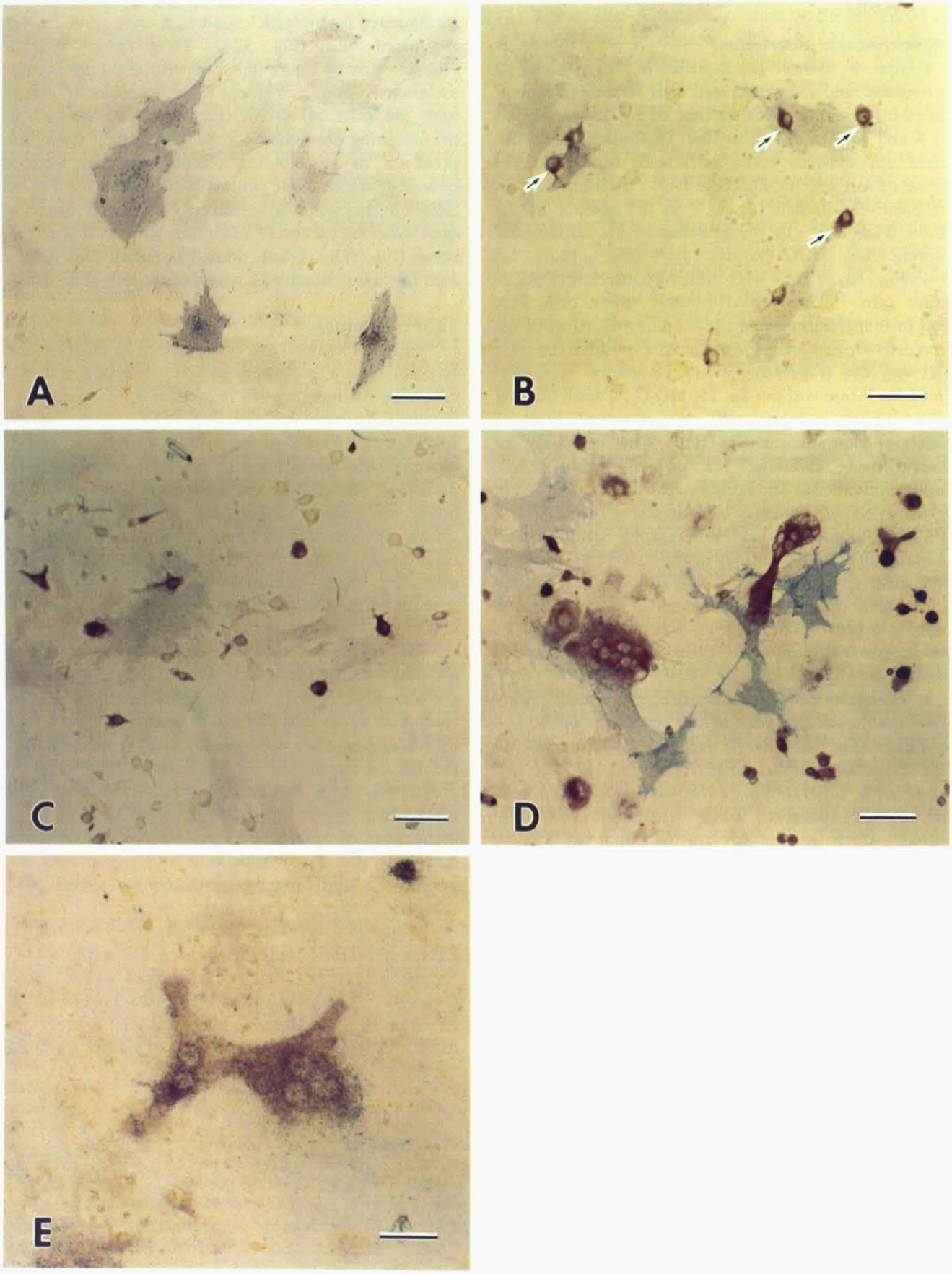

Fig. 1

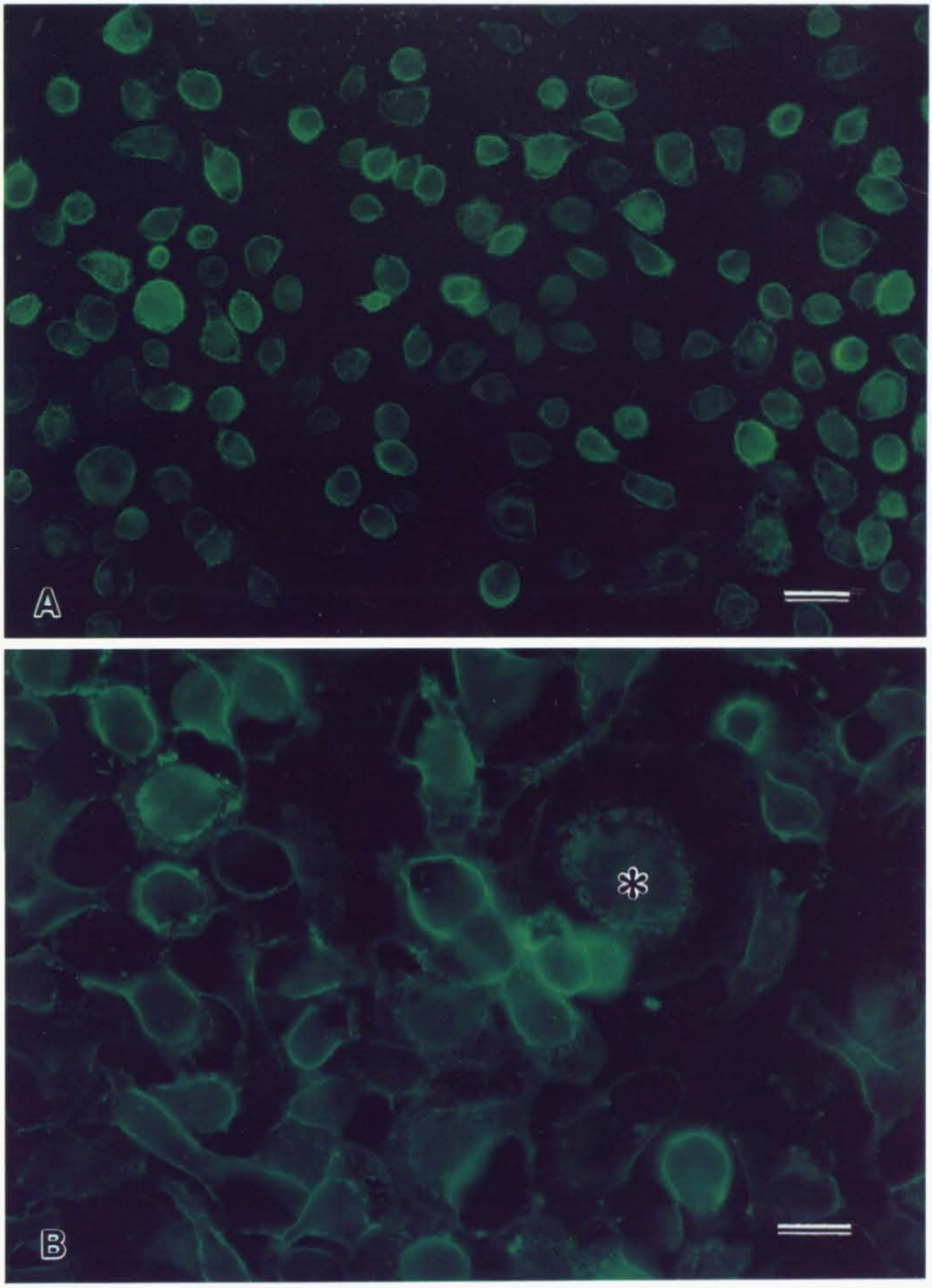

Fig. 2. Immunofluorescent examination for F4/80 which is specific marker for murine macrophage-monocyte lineage. **(A)** F4/80-immunopositivity is seen on the cell surfaces of round spleen cells on day 4. There is no immunoreactivity on the fattened osteoblastic cells. Original magnification = ×600. Bar = 15 μm. **(B)** On day 7, F4/80-immunopositive cells are abundant. Although the immunoreactivity is observed on the cell surface of round spleen cells, a spreading cell (asterisk) does not display the immunopositivity on the cell surface. Original magnification = ×1000. Bar = 8 μm.

formed coated pits, and clathrins were aligned along the inner side of the coated pits (Fig. 5B, C). Interestingly, short cytoplasmic processes were often inserted into the coated pits (Fig. 5D). In the pits, amorphrous electron-dense components consisting of organic materials were seen to be associated with the opposing cell membrane (Fig. 5E), suggesting the possibility that closely adjacent osteoblastic cells and spleen cells could form coated pits in the extracellular microenvironment. It is likely that the coated pits act for internalization of organic materials, i.e., receptor-mediated endocytosis.

Figure 6 shows cell-cell contact between two

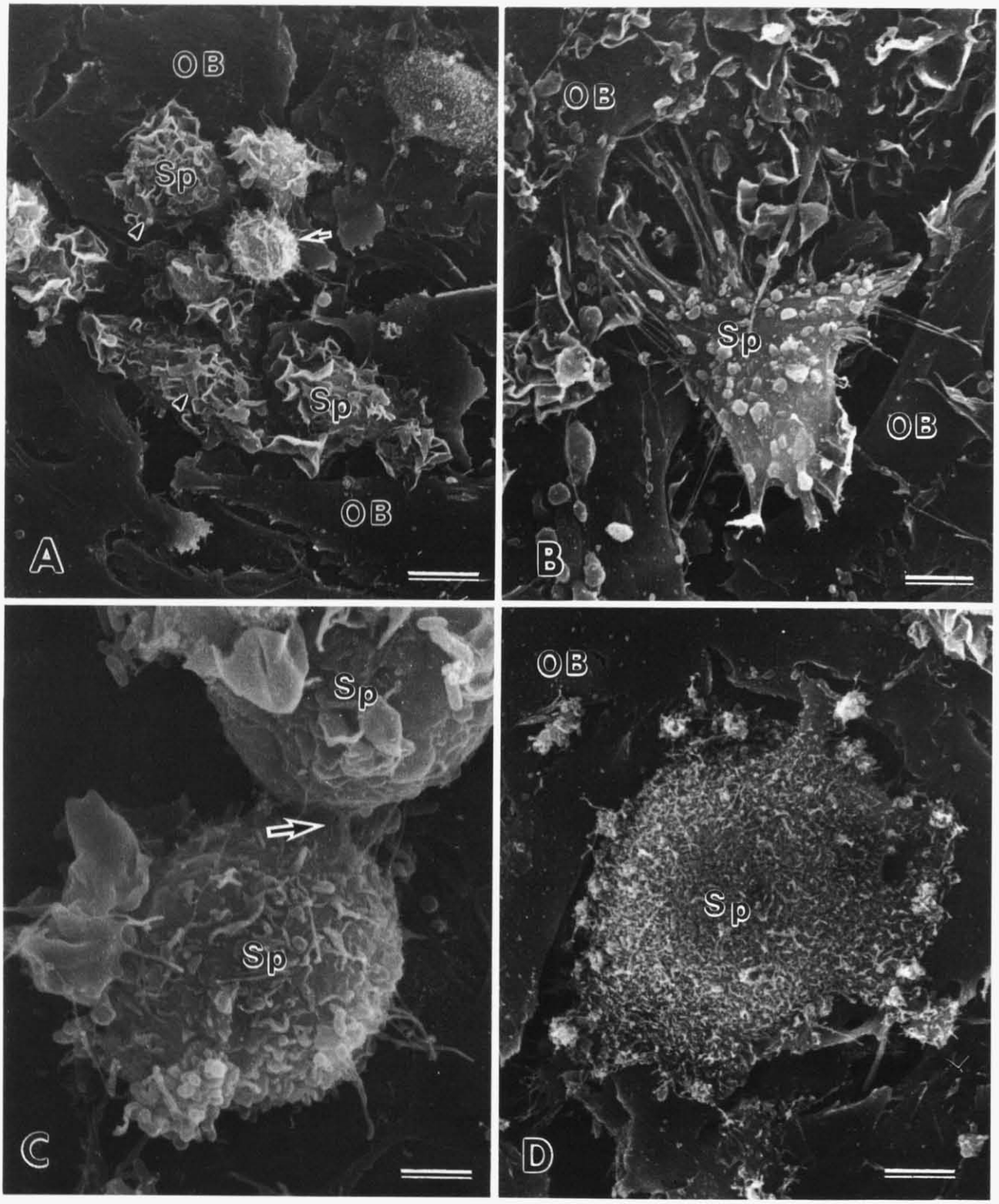

Fig. 3. SEM observations of co-cultures treated with 1α, $25(OH)_2D_3$ for 7 days. **(A)** Spleen cells (Sp) made clumps on the flattened osteoblastic cells (OB), showing numerous short cytoplasmic processess (arrow) and infoldings (arrowheads) on the cell surface. Original magnification = $\times 1200$. Bar = 8 μm. **(B)** A spleen cell extending long cytoplasmic filopodia to surrounding osteoblastic cells. The osteoblastic cells also extended cytoplasmic processes to the spleen cells. Original magnification = $\times 2{,}100$. Bar = 5 μm. **(C)** Two spherical spleen cells made contact with each other via their short cytoplasmic processess (arrow). Original magnification = $\times 5{,}200$. Bar = 2 μm. **(D)** A giant spleen cell formed in close association with osteoblastic cells. Original magnification = $\times 1{,}500$. Bar = 7 μm.

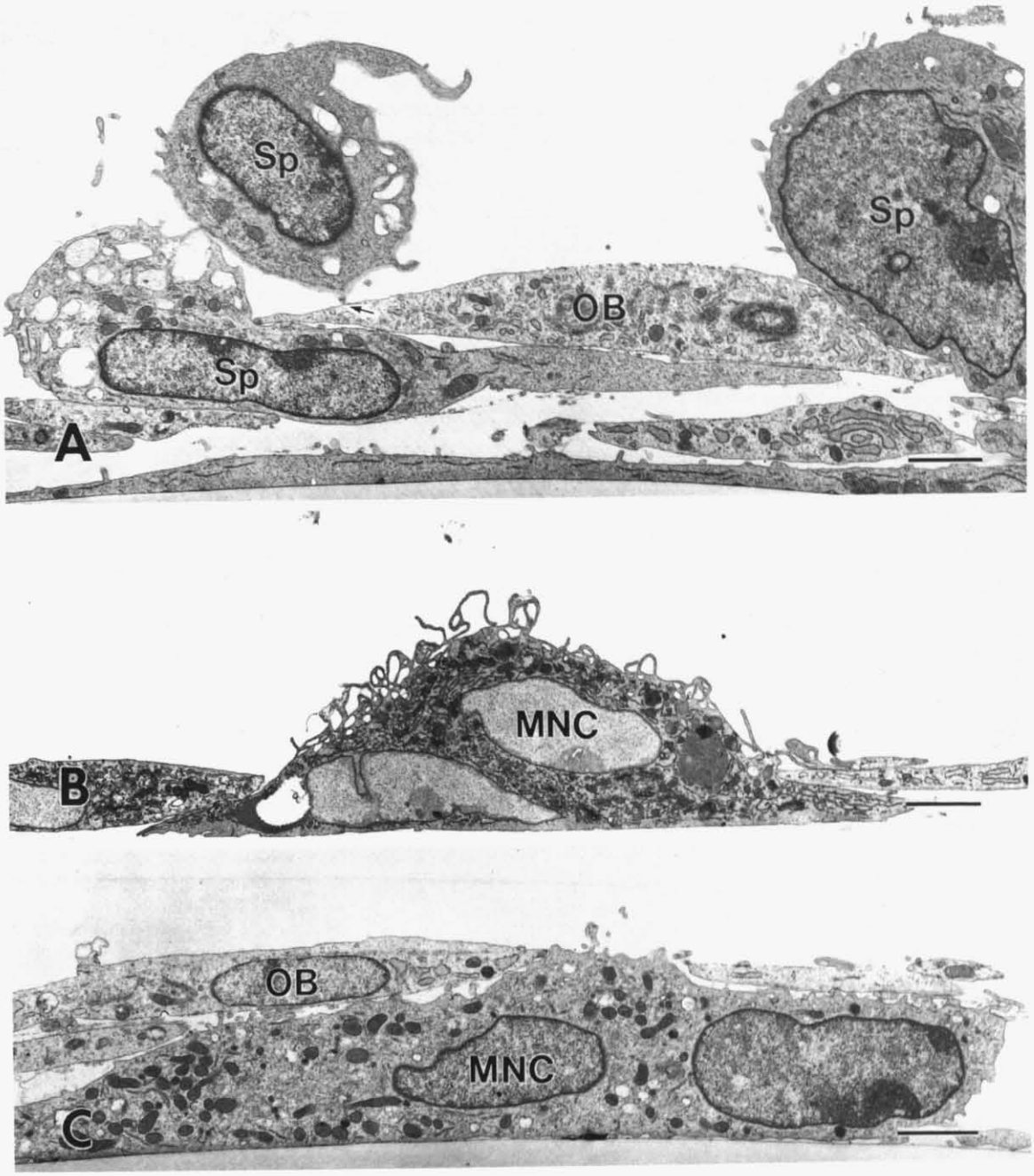

Fig. 4. TEM images of cross sectioned co-cultures at a low magnification. **(A)** A mononuclear spleen cell (Sp) began to make contact with a flattened osteoblastic cell (OB) by means of short cytoplasmic process (arrows). Another spleen cell extends its projection underneath the osteoblastic cell. Original magnification = ×4,300. Bar = 2.5 μm. **(B)** A multinucleated spleen cell (MNC) directly attaches to the coverslip in association with osteoblastic cells. Original magnification = ×3,800. Bar = 2.5 μm. **(C)** A flattened multinucleated cell having numerous mitochondria in the cytoplasm. Original magnification = ×4,500. Bar = 2 μm.

mononuclear spleen cells under TEM. When adjacent mononuclear spleen cells came to have many cell organelles, they appeared to easily make contact with each other. These organelles consisted of poorly-developed rough endoplasmic reticulum, several stacks of Golgi apparatus and numerous scattered mitochnodria in their cytoplasms. They also displayed coated pits and vesicles at cell-cell contact sites.

We also examined cell-cell contact between multinucleated gaint cells and mononuclear spleen cells (Fig. 7A). Mononuclear spleen cells sometimes extended their long cytoplasmic process to pits on the plasma membranes of the multinuclear giant cells (Fig. 7B). Multinucleated giant cells also showed infoldings of their cytoplasmic processes with those of spleen cells, presumably indicating cell-cell fusion between mononuclear spleen cells and multinuceated giant cells (Fig. 7C).

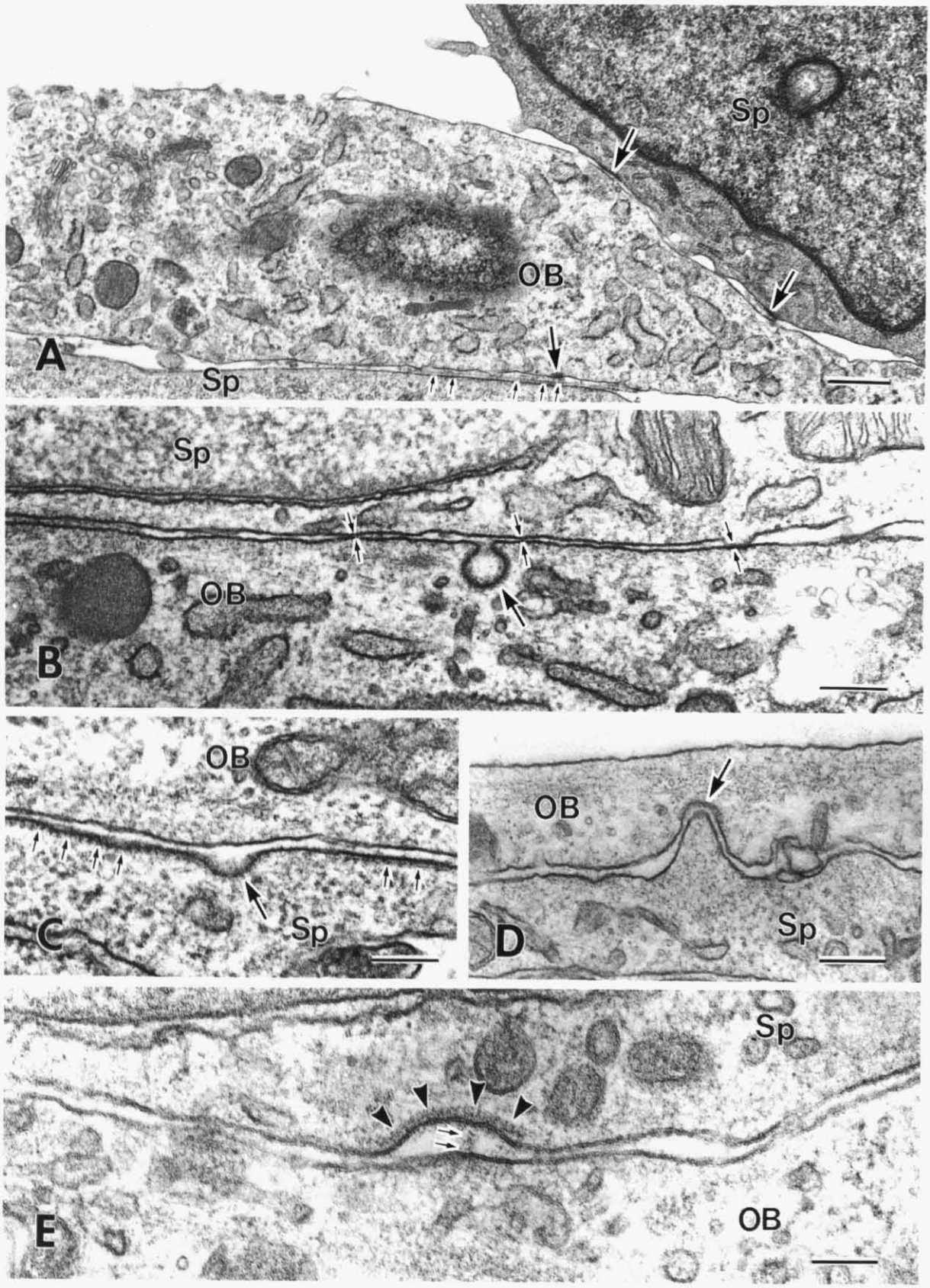

Fig. 5

IV. Discussion

The present study demonstrates fine structures of the cell-cell contact formed between osteoblastic cells and spleen cells in the culture model for osteoclast differentiation. The spleen contains a heterogenious cell population that includes red blood cells, macrophage-monocyte lineages and lymphocytes. However, spleen cells cultured on both day 4 and 7 revealed abundant F4/80-immunopositivity, indicating that most of them had somehow differentiated into macrophage-monocyte. In addition, neither lymphocyte, granulocytes nor red blood cells were identified in the co-culture under TEM observation. Therefore, spleen-derived cells appear to be of macrophage-monocytes lineage although the differentiation stage of these cells is not clear.

Autoradiography of [^{125}I]-elcatonin revealed that TRAP-positive multinucleated cells possessed abundant

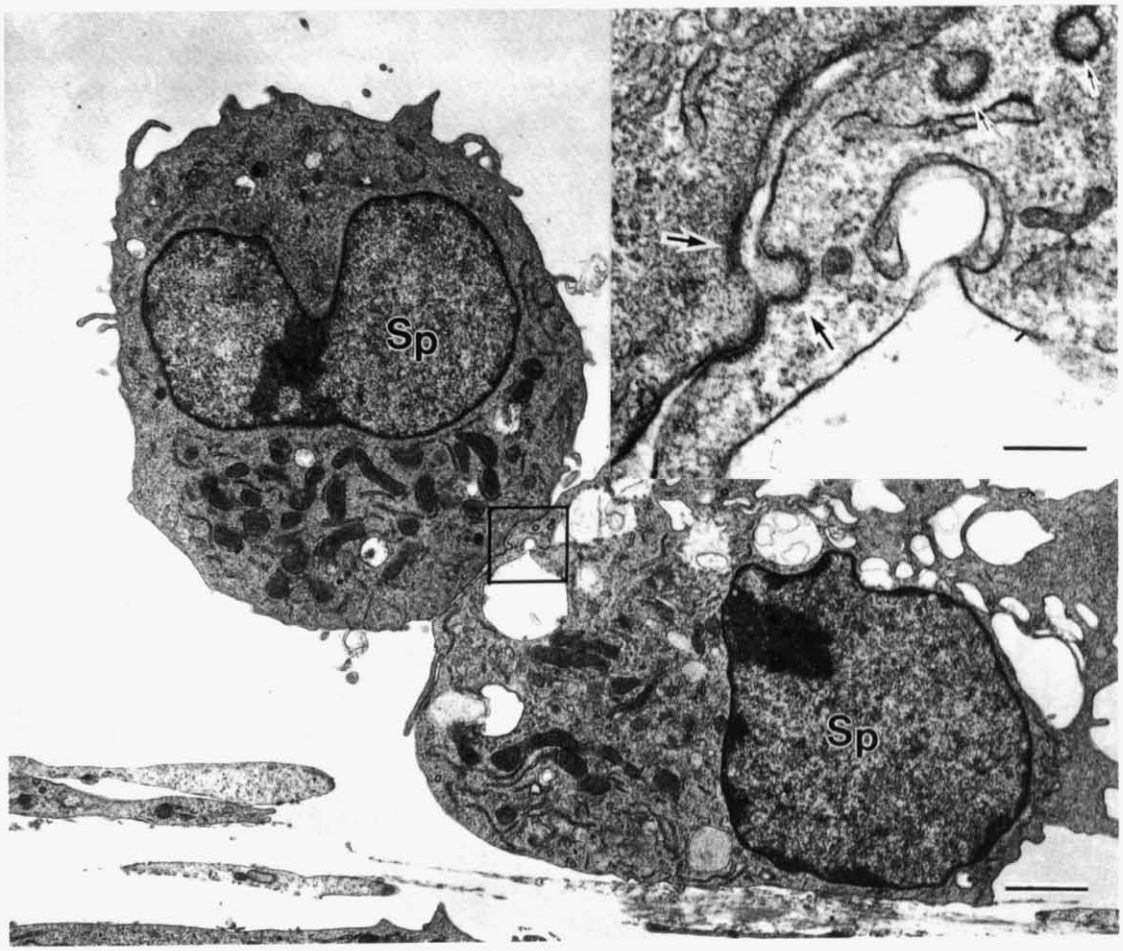

Fig. 6. Two spleen cells (Sp) which make contact with each other show well-developed intracellular organelles. Original magnification = ×5,200. Bar = 2 μm. Inset; Coated pits (large arrows) and coated vesicles (small arrows) were formed both on the cell membranes at the site of cell-cell contact. Original magnification = ×30,000. Bar = 0.3 μm.

Fig. 5. Ultrastructures of the cell-cell contacts between osteoblastic cells (OB) and spleen cells (Sp). **(A)** A high magnification of Figure 4A. When mononuclear spleen cells and osteoblastic cells make contact to each other, electron-dense cytoplasmic plaques appear on their inner plasma membranes (large arrows). Linear extracellular matrix accumulates in the narrow spaces between the osteoblastic cell and the spleen cell (small arrows). Original magnification = ×15,000. Bar = 0.8 μm. **(B)** Contact of the cell membranes was seen at different spots (small arrows); which consequently formed a closed extracellular microenvironment. A coated pit was formed on the plasma membrane facing to the closed extracellular microenvironment (large arrow). Original magnification = ×40,000. Bar = 0.25 μm. **(C)** Numerous clathrins were seen on the plasma membrane (small arrows). A coated pit is observed in the vicinity of clathrins alined on the cell membrane (large arrow). Original magnification = ×38,000. Bar = 0.25 μm. **(D)** A short cytoplasmic process is inserted into the coated pit. Original magnification = ×42,000. Bar = 0.25 μm. **(E)** Amorphous electron dense components are seen faintly along the coated pit (arrowheads). Organic components (small arrows) are not only bound to the coated pits but also associated with the opposite cell membrane. Original magnification = ×90,000. Bar = 0.1 μm.

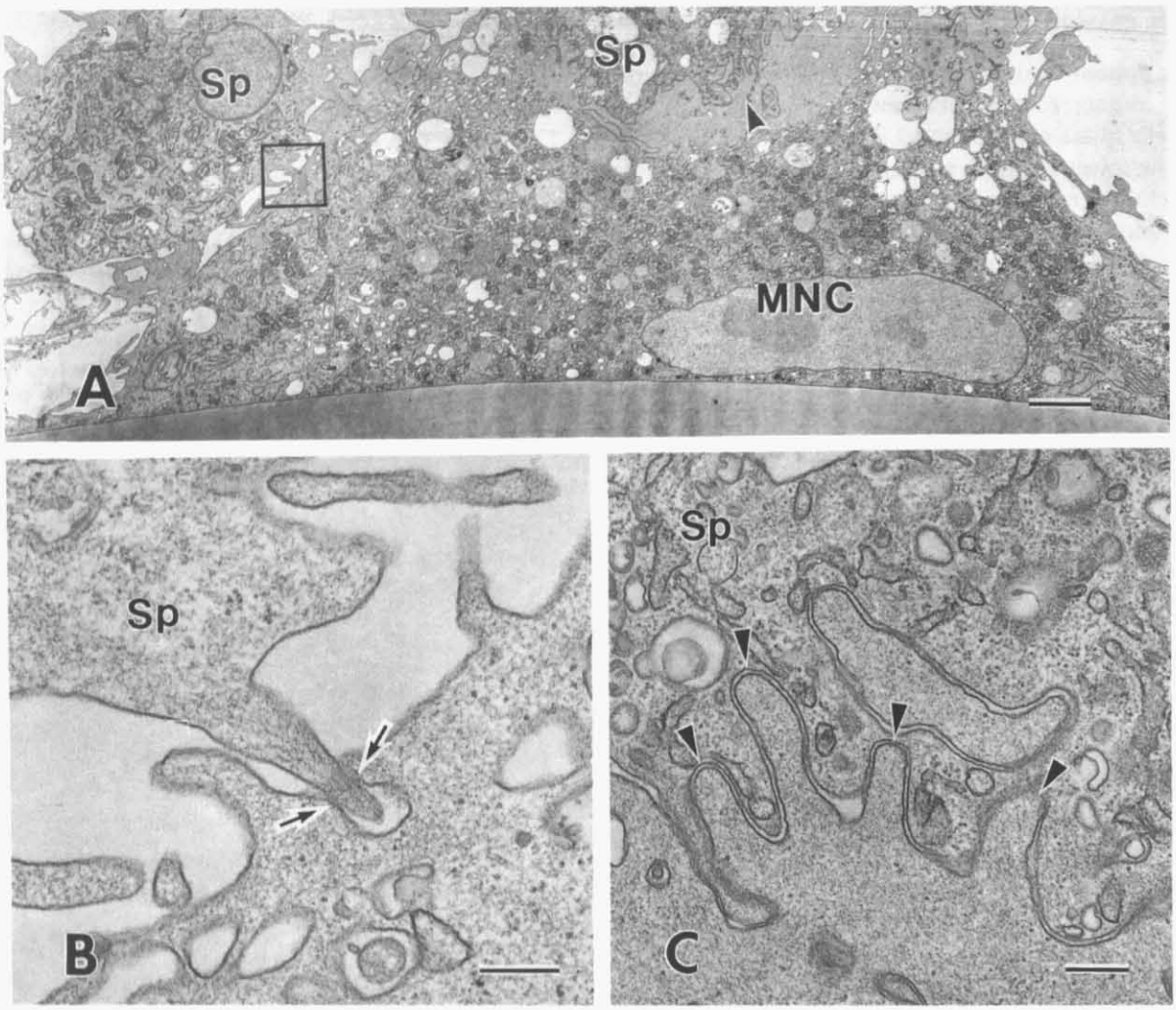

Fig. 7. Cell-cell contact between multinucleated giant cell (MNC) and mononuclear spleen cells (Sp). **(A)** Two spleen cells attach to the giant multinuceated cells. Original magnification = ×3,200. Bar = 3 μm. **(B)** Higher magnification of a square in Fig A. A cytoplasmic process of a spleen cell inserts into a pit on the cell membrane of the multinucleated giant cell, showing menbrane contacts between the cytoplasmic process and the pit. Original magnification = ×21,000. Bar = 0.5 μm. **(C)** Higher magnified image of the contact site indicated by arrowhead in Fig. A. Infoldings of cytoplasmic processes of the multinucleated giant cell with those of a spleen cell can been seen (arrowheads). There are numerous vesicles in the cytoplasm adjacent to contact site, whereas few cell organelles are present in the cytoplasm of multinucleated cells. Original magnification = ×17,000. Bar = 0.5 μm.

and specific binding sites for elcatonin, satisfying the major criteria for authentic osteoclast [4, 17, 30]. Double-staining for ALP and TRAP activities revealed that only spleen cells in contact with ALP-positive osteoblastic cells became TRAP-positive. 1α, $25(OH)_2D_3$ greatly stimulated and accelerated the conversion. These results confirmed our previous concept that osteoblastic cells are a prerequisite for osteoclast differentiation of spleen cells. Cell-cell contact between the two types of cells appeared essential for the commitment of spleen cells into osteoclast differentiation. Interestingly, spleen cells making solid contact with dead osteoblastic cells fixed with glutaraldehyde failed to become TRAP-positive [31], indicating that cell-cell recognition between living spleen cells and osteoblastic cells is essential. 1α, $25(OH)_2D_3$ facilitated fusion and TRAP activity in spleen cells, but a small number of TRAP-positive spleen cells appeared in the co-cultures without the addition of 1α, $25(OH)_2D_3$. This may be due to the presence of 1α, $25(OH)_2D_3$ and other bone-resorbing hormones and cytokines in FBS used in this study.

TEM images at a low magnification revealed that mononuclear spleen cells tended to migrate under osteoblastic cells in association with them. Such behavior of spleen cells in this co-culture system seems to be identical with authentic preosteoclasts, which migrate toward bone surfaces in contact with osteoblastic cells [6, 20]. Furthermore, the ultrastructure of multinucleated spleen cells displayed a number of similarities to those of authentic osteoclasts [1, 10]. Thus, it is likely that multinucleated cells formed in this co-culture system and authentic osteoclasts employ a similar differentiation pathway.

SEM observations revealed that spleen cells were capable of interacting with relatively distant osteoblastic cells by extending filopodia. Long cytoplasmic processes may also be useful for spleen cells and osteoblastic cells to recognize neighbouring cells. Therefore, the TRAP-positive spleen cells formed in the vicinity of ALP-positive osteoblastic cells seen under light microscopy probably make direct contact with osteoblastic cells.

TEM observations demonstrated fine structures at the sites of cell-cell contact. Osteoblastic cells and spleen cells exhibited electron-dense cytoplasmic materials possibly adherens junctions, [9, 23] on the inner leaflets of plasma membranes (Fig. 5A). These adherens junctions may be localized on the plasma membranes of neighbouring spleen cells and osteoblastic cells, so as to expedite the establishment of focal contacts between cell membranes (Fig. 5B-E). Considering the origin of osteoblastic cells (mesenchymal cells) and spleen cells (haematopoietic cells), it is likely that adherens junctions could be formed between heterogeneous, as well as homogeneous types of cells. Since adherens junctions were sometimes seen on the inner cell membrane coated with linear extracellular matrix, spleen cells and osteoblastic cells could also become attached to each other by involving extracellular matrix.

The ultrastructure at the cell-cell contact sites demonstrated a narrow intercellular microenvironment between osteoblastic cells and spleen cells. Since the microenvironment is enclosed within a virtual "wall" of contact between cell membranes, organic components associated with the cell membrane could be retained intact. Within the microenvironment, amorphous materials present on the membranes of osteoblastic cells were associated with the coated pits on the corresponding spleen cell (Fig. 5). Organic materials present in the microenvironment would therefore be internalized by the coated pits and coated vesicles in spleen cells. Based on the experimental fact that the co-culture of dead osteoblasts with live spleen cells failed to generate osteoclasts, it may be possible to speculate that osteoblastic cells recognize spleen cells, then provide necessary information or materials for osteoclast differentiation at the cell-cell contact site. The micro-environment appears to function as an active site for intercellular transport of organic materials.

Many cells may form contacts with other types of cells to induce cell differentiation. For instance, marrow stromal cells have been reported to bind exogenous granulocyte and macrophage-colony stimulating factor (GM-CSF) via glycosaminoglycans produced by stromal cells, and support differentiation of haematopoietic stem cells [11]. To date, macrophage colony-stimulating factor (M-CSF) seems to be one of strong candidates for modulation of osteoclast differentiation [8, 14, 15, 26]. Since M-CSF has been reported to bind an extracellular matrix as a proteoglycan form [25], further morphological assessment will be promissing for elucidating the biological function of M-CSF at the cell-cell or cell-matrix contact sites during osteoclast differentiation.

In this study, no gap junctions were observed between osteoblastic cells and spleen cells, although osteoblastic cells possessed gap junctions between themselves (data not shown). Indeed, authentic osteoblasts have gap junctions between preosteoblasts and osteoblasts/osteocytes [5, 21]. These gap junctions may play an important biological role in cell-cell communication, as previously shown in cyclic AMP and Ca^{2+} transport into neighbouring cells [16]. It could be speculated that the lack of gap junctions between osteoblastic cells and spleen cells may be due to the heterogeneous origin.

Coated pits were also formed at the site of cell-cell contact between two spleen cells. The clathrin-bound coated pits have been reported as morphological evidence for cell-cell contact in the initial step of the fusion process of alveolar macrophages [7]. The present study shows ultrastructural features at intercellular contact sites of spleen cells similar to those of alveolar macrophages. Multinucleated cells also displayed numerous pits and vesicles at contact sites with mononuclear spleen cells, representing morphological evidence for cell-cell communication prior to cell fusion (Fig. 7).

In summary, the present study provides morphological features of cell-cell contact between spleen cells and osteoblastic cells. Formation of a microenvironment at the site of cell-cell contact appears to be directly involved in the internalization of organic components, which may be a prerequisite for osteoclast differentiation.

V. Acknowledgments

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