DETECTION OF GAP JUNCTIONS BETWEEN THE PROGENY OF A CANINE MACROPHAGE COLONY-FORMING CELL IN VITRO

MARTIN PORVAZNIK and THOMAS J. MACVITTIE

From the Experimental Hematology Department, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20014

ABSTRACT

An in vitro monocyte-macrophage colony-forming cell (M-CFC) has been detected in canine bone marrow (BM). The colonies derived from these progenitor cells were similar to murine-derived M-CFC (MacVittie and Porvaznik, 1978, J. Cell Physiol. 97:305-314) colonies, since they showed a singular macrophage line of differentiation, a lag of 14–16 days before initiating colony formation, and they survived significantly longer in culture in the absence of colony-stimulating factor (CSF) than granulocyte-macrophage colony-forming cells (GM-CFC). Endotoxin (Salmonella typhosa lipopolysaccharide W)-stimulated dog serum was used as the CSF (7% vol/vol). Canine-derived M-CFC progeny were identified as macrophages on the basis of morphology, phagocytosis, and the presence of Fc receptors for IgG. Gap junctions were observed only in canine BM, M-CFC-derived colonies using freeze-fracture and lanthanum tracer techniques. They were not observed in any GM-CFC-derived colonies. The number of gap junctions observed in freeze-fracture replicas of BM, M-CFC-derived colonies (21 colonies from three different dogs) showed a significantly positive correlation (Kendall’s $\tau = 0.70$, $P < 0.001$) with the size of the colony fracture plane area. Gap junctions were observed displaying hexagonal lattices of 9.3 nm ± 0.08 (SE) particles with a center-to-center spacing of 10.4 nm ± 1.0 (SE) on membrane P-fracture faces. On membrane E-fracture faces, highly ordered arrays of pits with 8.7 nm ± 0.12 (SE) center-to-center spacing were observed. Arrays of both particles and pits were also observed in fracture-face breakthroughs within a gap junction. Thus, gap junctions can form in vitro between the cells of macrophage progeny of a canine M-CFC under appropriate growth conditions. The significance of this observation is that there may be a structural basis for cell-to-cell collaboration between BM macrophages and other capable cells that either pass into the tissue for modification or develop there into mature cell forms.

KEY WORDS macrophage · colony-forming committed to the macrophage line of differentiation (14). These M-CFC’s were detected in the cell · gap junctions bone marrow, spleen, and peripheral blood leukocytes (14). Here we report the detection of these

We have recently reported the detection of a murine in vitro colony-forming cell (M-CFC) solely derived from canine bone marrow (BM).
The morphology of the cellular interactions between macrophage progeny within these M-CFC-derived colonies is of considerable interest to us, since tissue macrophages may act as "helper" cells in a variety of situations (1, 7, 29). One type of cellular interaction is based on the nexus or gap junction. This structure has been implicated as the structural pathway for cell-to-cell communication and metabolic cooperation (for reviews, see references 5, 19, and 28). Electron microscope observations of gap junctional membranes that are detected by the freeze-fracture technique reveal a unique organization of hexagonally packed intramembrane particles, which are generally organized in circular plaques. During the formation of gap junctions, it has been suggested that the junctional particles in one membrane may be matched with another set of intramembrane particles in the adjacent cell (9). Each junctional particle has been reported to have a 2-nm channel (16, 27), permitting the transfer of ions and molecules with a discrete size and geometry (27). Cells that have been found to be linked by gap junctions and that transfer dye molecules intercellularly (22) have also been found to be electrically coupled (8). These observations strongly implicate gap junctions in providing a private pathway for cellular communication and cooperation.

The present study shows unequivocally that macrophages derived from BM marrow cells, and the cell colonies derived from BM marrow colonies, will develop gap junctions when cultured under specific growth conditions. The significance of this observation is that this may provide a structural basis for cell-to-cell collaboration between BM macrophages and other capable cells that either pass into the tissue for modification or develop there into mature cell forms.

MATERIALS AND METHODS

Animals

Healthy, purebred, male beagles, weighing 10–15 kg, were used in this study. A veterinarian treated the dogs to eliminate parasite infections and immunized them against distemper, hepatitis, and rabies. They were observed for 2–3 wk before being used. They were housed in temperature-controlled rooms, in individual stainless steel cages, and fed kibbled laboratory dog food, supplemented once a week with high-protein, canned-meat ration. Water was provided ad lib.

Agar Cultures

The double-layer agar culture technique used was similar to that described previously (15), except that dog serum collected after stimulation with bacterial endotoxin was used as the source of colony-stimulating factor (CSF) (7% vol/vol). Dog serum was harvested 4 h after an i.v. injection (cephalic vein) of 50 μg of endotoxin from Salmonella typhosa (Bacto Lipopolysaccharide W, Difco Laboratories, Detroit, Mich.) dissolved in pyrogen-free saline (15).

Bone marrow, from the ribs of anesthetized dogs (Surtwal, Parke Davis, A. J. Buck & Son, Baltimore, Md.), was aspirated into a syringe containing preservative-free heparin (20 U/ml). Nucleated bone marrow cells (BMC) were separated from the BM aspirate by sedimentation in Eagle's minimal essential medium with 3% dextran. The cells were washed twice and suspended in McCoy's 5a medium with 10% fetal calf serum for cell counting. Marrow cells in suspension were plated in triplicate at a concentration of 2 × 10^5 BMC/dish (25 × 10 mm style, Falcon Labware, Div. of Becton, Dickinson & Co., Oxford, Calif.) and incubated at 37°C in a humidified, 5% CO_2 in air atmosphere. Colonies (>50 cells) counted after 27 d were considered to be derived from M-CFC. Colonies counted after 8 d of culture were considered to be derived from a granulocyte-macrophage colony-forming cell (GM-CFC). These GM-CFC are sensitive to the absence of CSF in the culture, and a large percentage lose their proliferative ability within 48 h. Therefore, to determine the survival of GM-CFC and M-CFC in the absence of CSF, the BMC-derived cell cultures were prepared without CSF. At selected days after incubation, CSF was added to the culture dish in a 0.5-ml aliquot of 0.33% agar medium mix. Control cultures were initiated immediately after they had jelled. Respective cultures were then incubated for an additional 8 and 28 d after addition of CSF.

IgG Receptors

Antiserum to sheep erythrocytes was obtained from N. L. Cappel Laboratories Inc. (Cochranville, Pa.). Equal volumes of 2% (vol/vol) sheep erythrocyte and 10% (vol/vol) heat-inactivated rabbit anti-sheep erythrocyte serum in McCoy's 5a were mixed and then incubated for 30 min at 37°C (6). IgG-coated sheep erythrocytes were then washed and resuspended in McCoy's 5a. Rosette formation was studied in cells derived from 27-d colonies grown in agar culture. These cells were removed from agar culture, dispersed with a sterile needle and syringe in McCoy's 5a/10% fetal calf serum, and pipetted onto Thermopax tissue culture cover slips (Lux Scientific Corp., Newbury Park, Calif.). They were placed in sterile Petri dishes and allowed to attach overnight at 37°C. The viability of cells derived from 27-d agar colonies was 94% by trypan blue exclusion. These adherent cells derived from agar cultures were washed two times with McCoy's 5a medium just before rosette formation. IgG-coated sheep erythrocytes were added (10^9/ml) to the cultures in 1 ml McCoy's 5a and incubated for 30 min at 37°C. Control cultures were initiated.
with washed sheep erythrocytes, not complexed with IgG. The cultures were then washed three times with McCoy's 5a medium, and processed for scanning electron microscopy (SEM).

Phagocytosis

Cells derived from 27-d colonies grown in agar were prepared as described above. Log phase Escherichia coli were added to McCoy's 5a containing 20% dog serum. Aliquots were added to the cultures to obtain an approximate bacteria-to-leukocyte ratio of at least 10:1. After 60-min incubation at 37°C, the plates were washed twice to remove noningested bacteria, then air-dried and stained with Wright's-Leishman stain for examination. Phagocytosis of IgG-coated sheep erythrocytes was observed by prolonging the incubation used for rosette formation to 60 min. Thereafter, the culture plates were treated in the same manner for fixation, staining, and examination.

Cell Morphology and Electron Microscopy

Colony cell morphology was determined by bright-field, phase-contrast, and electron microscopy. M-CFC-derived colonies were fixed in situ with a glutaraldehyde-parafomaldehyde mixture (10). Individual colonies were carefully removed from the culture dishes with a Pasteur pipette and transferred to vials for postfixation in collidine-buffered osmium tetroxide, uranyl acetate staining (11), ethanol dehydration, and Epon embedment. Colonies were also prepared for electron microscopy with lanthanum tracer, using the method of Revel and Karnovsky (23). 1- to 1.5-μm-thick sections were cut for light microscopy, poststained for 30 s with methylene blue, mounted in permount (Fisher Scientific Co., Pittsburgh, Pa.), and examined with a Zeiss Ultraphot II microscope (Carl Zeiss, Wuertemberg, West Germany). Thin sections were cut with a diamond knife (Rondikin Corp., Honolulu, Hawaii) on a Reichert OmU3 ultramicrotome (Vienna, Austria), stained briefly with uranyl acetate and lead citrate (24) if desired, and examined in a Philips 400 electron microscope (Eindhoven, The Netherlands).

For freeze-fracturing (F-F), M-CFC-derived colonies were adequately fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate, pH 7.2, rinsed in buffer, cryoprotected in 30% glycerol buffered with 0.1 M cacodylate, frozen in gold holders, and fractured by reported methods for the Balzers 360M F-F device (21) (Balzers High Vacuum Corp., Lichtenstein) equipped with an electron gun.

For SEM, adherent cells derived from agar cultures were grown on Thermax cover slips, fixed, and dehydrated as described above. After the third change in 100% ethanol, the cover slips were placed in a Samdri PVT-3 critical point drying apparatus (Tousimis, Rockville, Md.) using CO₂ as the transitional fluid. Dried specimens were subsequently placed in a Samsputter 2A (Tousimis) and coated with gold-palladium. Coated specimens were viewed in an ETEC autoscan (Hayward, Calif.).

A total of 21 colonies originating from three different dogs were freeze-fractured and replicated. Replicas were scanned at ×12,500 for intercellular junctions while viewed through ×10 binoculars. Membrane faces were labeled as either protoplasmic fracture faces (P face) or exoplasmic fracture faces (E face) (2). All micrographs were oriented approximately with the direction of platinum shadowing from the bottom, unless otherwise indicated by an encircled arrow.

Measurement of Particles and Spacings

Before each gap junction was photographed, the replicas were adjusted for specimen height and placed at 0° tilt so that the specimen and focal plane were oriented perpendicular to the axis of the electron beam. Particle sizes in F-F replicas were measured perpendicular to the direction of shadowing from point-to-point on the crescent-shaped accumulation of platinum. The values given for particle sizes and spacing were calculated from measurements made on enlarged micrographs (×64,500) and currently calibrated negative magnifications (×21,500).

RESULTS

Growth of GM-CFC- and M-CFC-Derived Colonies from BM

GM-CFC-derived colonies reached maximum numbers within 8 d after initiation of culture. Endotoxin-stimulated dog sera used as the source of CSF promoted an earlier initiation of colony formation and an average number of 128 colonies (SEM ± 25) per 10⁵ nucleated BMC cultured (range, 64–290 per 10⁵ BMC). The colonies disintegrated quickly with increased time of incubation. Interestingly, we could occasionally observe the initiation of a presumptive M-CFC-derived colony within the center of a disintegrating GM-CFC-derived colony.

Mononuclear-macrophage colonies considered to be derived from an M-CFC exhibited several characteristics similar to those at the M-CFC-derived colonies in murine tissues (13, 14). Colonies derived from M-CFC initiated growth only after a significant lag period of 14–16 days of culture and reached maximum numbers by 25–28 d of culture. Wright-Giemsa-stained smears of cells within colonies removed at various times from culture revealed only mononuclear cells and macrophages. No granulocytes were observed within any colony.
The number of M-CFC detected within canine BMC aspirates ranged from 82 to 600 per 10^5 nucleated cells cultured, with a mean value of 277 (SEM ±48) per 10^5 cells representing 12 separate experiments. M-CFC were approx. 2.2-fold greater in concentration than GM-CFC over the same experiments. When BMC were cultured within the range 2 × 10^4 to 2 × 10^5 nucleated cells, a linear response was observed in M-CFC-derived colony formation. Linearity was reduced within high-growth cultures at the upper cell concentrations, perhaps due to crowding and nutrient limitations.

**Survival of Colony-Forming Cells in the Absence of CSF**

Canine M-CFC were significantly more resistant to the absence of CSF in culture than the GM-CFC (Fig. 1). 24 h without CSF reduced GM-CFC-derived colonies to ~50% of control, whereas M-CFC-derived colonies were decreased only by ~5% of control. It required 3 d of culture in the absence of CSF to reduce M-CFC to an equivalent 50% of control.

**Phagocytosis and Fc Receptors of M-CFC-Derived Cells**

The majority of cells within colonies showed a marked degree of phagocytized and pinocytized agar. Adherent cells from 28-d colonies were capable of ingesting 10-30 E. coli per cell. When IgG-coated sheep erythrocytes were added to the adherent cells derived from day-28 agar colonies, ~92% of all cells were covered with sheep erythrocytes and formed rosettes. Prolongation of the incubation time with sheep erythrocytes resulted in a marked degree of erythrophagocytosis.

**Colony Morphology**

Phase-contrast microscopy of intact colonies grown in agar showed that most cells appeared to be in very close contact with each other, especially in the center of the colonies (Fig. 2). Stained thick sections through M-CFC-derived colonies showed a looser organization of cells than expected, although cells in contact were always observed (Fig. 3). Both light and electron microscopy showed that the majority of cells within a colony were irregular in shape, with eccentrically placed oval nuclei. Most cells had well-developed cytocenters containing extensive endoplasmic reticulum, vesicles, vacuoles, and Golgi bodies (Fig. 4).
Particle diameter was 9.3 nm ± 0.08 (SE) (245 measurements from 41 gap junctions). The mean center-to-center spacing of the gap junctional particles was 10.4 nm ± 1.0 (SE) (231 measurements from 39 gap junctions). Membrane E-fracture face views of the macrophage gap junctions revealed highly ordered arrays of pits with a center-to-center spacing of 8.7 nm ± 0.12 (SE) (85 measurements from 21 different gap junctions).

Lanthanum tracer preparations were used to more easily detect gap junctions in thin sections of M-CFC-derived colonies. Although thinly sectioned specimens showed less convincing gap junctions, the highly ordered spacing in tangential sections was present.

In many thin sections of M-CFC-derived colonies, small processes could be observed abutting adjacent cells, yet no distinct gap junctions were present (Fig. 6 and inset). However, since the freeze-fracture technique revealed a greater area of the abutting intercellular processes, gap junctions in such areas were readily observed (Fig. 7).

**DISCUSSION**

Canine BMC, when cultured in the presence of an appropriate CSF, will form macroscopic colonies within 25–28 d, composed entirely of mononuclear phagocytes or macrophages. These macrophage progeny are presumed to be derived from a single colony-forming cell, M-CFC, present within the BMC suspension. The marrow-derived M-CFC are most probably the precursors of the free, mobile macrophages of the mononuclear phagocyte system, and are probably capable of seeding the extramedullary organs and tissue spaces that are included within the mononuclear phagocyte system. M-CFC have also been detected within the canine peripheral blood mononuclear cells and peritoneal exudate cells. However, we have not detected any gap junctions in colonies derived...
Irregular cell shapes and eccentrically situated oval nuclei (N) are observed in freeze-fracture replicas of M-CFC-derived colonies. Nuclear:cytoplasmic ratios are <1. Most cells have well-developed cytocenters containing many vesicles and larger phagocytic vacuoles. One striking feature of these cultured macrophages is the presence of intercellular gap junctions (arrow, see inset). Bar, 2.5 μm × 8,150. Inset: Higher magnification of the gap junction observed in Fig. 4. Notice the close hexagonal packing of the intramembrane particles that comprise the gap junction, × 41,900.
from peripheral blood. We have not yet examined those colonies derived from peritoneal exudate (unpublished observations). The macrophage progeny derived from the M-CFC were identified morphologically as well as by the presence of Fc receptors for IgG molecules, avid phagocytosis of E. coli bacteria and IgG-coated sheep erythrocytes, and glass adherence.

In this report, we present evidence that the macrophage progeny are linked intercellularly by gap junctions. These areas of intercellular connection have been strongly implicated as the structural pathways for cell-to-cell communication by transfer of informational molecules with a mol wt not greater than 1,660 daltons (27). The number of gap junctions between macrophages increased linearly as the colony fracture plane area increased. This may reflect a greater need for cell-to-cell cooperation where competition for nutrients is greater in the center of the colony than at the periphery of the colony where a greater cell area is exposed to the culture medium.

The importance of gap junctions between BM-derived macrophages is not known and can only be speculated on at this time. However, several important cell associations between macrophage-like cells and heterologous cell types exist, suggesting a functional collaboration. In the BM, erythroblastic "islets" are composed of a central macrophage or reticulo-histiocyte surrounded by differentiating erythroid cells that appear to be in the same stage of development (1). Plasma cells, whose principal function is to secrete gamma globulin (particularly antibody globulins), are often arranged in islets around a central macrophage or reticulo-histiocyte (29), and may be found in the bone marrow. Finally, some authors hypothesize information exchange between contacting lymphocytes and a central macrophage in lymphocytic islets (17, 18, 25, 26). Apparently, the contact event is essential to the activation of the lymphocytes. In all these collaborative events, the central macrophage appears to be required, and a cell contact event either coordinates or initiates a secondary event. Although a structural basis for the collaborative events has not been identified by routine electron microscope techniques, we believe that a reexamination of these various islets should be undertaken using the freeze-fracture technique to unequivocally identify any membrane specializations if they exist.

Recently, Levy et al. (12) presented evidence that cultured macrophages from various mouse tissues demonstrated electrotonic coupling only between cells in contact. They suggested that macrophages may communicate among themselves for certain normal functions. However, they failed to observe any definitive intercellular junctions. We suggest that our structural observations support the hypothesis that gap junctions are the structural basis for electronic coupling between cultured macrophages.

In another study, Gallin and Gallin (4) observed membrane hyperpolarizations and decreased membrane resistance in human cultured macro-

Figure 5 Fracture-face transitions within gap junctions often reveal both pits (arrow) on the membrane E face and particles on the membrane P face. Bar, 0.3 μm × 67,200.
In many thin sections of M-CFC-derived colonies, long macrophage processes may be in very close opposition (arrows), yet no distinct gap junctions are present even after tilting the sections within the electron beam. Bar, 2 μm × 14,050. Inset: Enlargement of "boxed" process abutting another cell. Notice that the extracellular space is clearly visible. × 69,900.
Since the freeze-fracture technique reveals a greater area of the abutting macrophage cell processes, gap junctions (arrow) in such areas are easily observed. Bar, 0.5 µm. × 41,250.

phages when in the presence of E. coli endotoxin-activated serum. Since the CSF in our cultures of M-CFC-derived colonies is also an endotoxin-activated serum, we suggest that gap junction interactions between macrophages may also be directed by chemotactic factors and may be essential for junction formation.

The colonies derived from GM-CFC did not show any detectable gap junction structures between the cells of the progeny. These colonies, however, are initially composed primarily of granulocytes with the gradual appearance of mononuclear cells and macrophages as the colony reaches maximum size (8–10 d in culture). This growth pattern approximates that observed previously by Marsh et al. (20). All colonies examined in our laboratory were of mixed morphology and were diffuse in appearance. The mixed nature of the colony as well as the diffuse pattern shown in agar may preclude the formation of gap junctions.

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