Formation of Capillary Networks from Bone Marrow Cultured in Collagen Gel

Masaharu Mori, Yoshito Sadahira*, Seiji Kawasaki, Toshiko Hayashi and Michiyasu Awai

*Department of Pathology, Okayama University Medical School, Okayama 700 and *Department of Pathology, Kawasaki Medical School, Kurashiki 701-01, Japan*

ABSTRACT. The growth of capillaries from mouse bone marrow was studied in collagen gel. When the culture contained sufficient bone marrow cells (more than 1×10^6 cells) and cell clusters, short capillaries with lumina appeared about one to two weeks after inoculation, following the proliferation of fibroblastic cells and hemopoietic cells. Four weeks after inoculation, these capillaries formed a network among hemopoietic cells and adipocytes.

Electron microscopic observations revealed that these capillaries had thin walls and poorly developed basement membranes, similar to the sinusoids of bone marrow. These capillaries did not appear when the amount of inoculated bone marrow was reduced or dispersed to the point that the marrow cell clusters disappeared. The quantity of the inoculum and the clustering of cells, therefore, seems to play important roles in the appearance of the capillaries.

Three-dimensional gel is a proven substrate for the examination of morphogenesis *in vitro*. Several cell types have shown three-dimensional organoid growth in collagen gel (8, 9, 15). Most notably, the angiogenetic process has been studied in detail using this gel. Montesano et al. reported that capillary endothelial cells cultured on the surface of collagen gels reorganized into capillary-like tubes when they were covered with a second layer of collagen (8). Furthermore, capillary outgrowth from muscular and adipose tissue was observed in collagen gel and fibrin clot (9). Nicosia et al. used a histophysiologic gradient culture with a fibrin clot and plasma clot and observed capillary growth from the end of an aortic segment as well as cavity formation in the aortic segment (13).

Concerning the culturing of bone marrow in collagen gel, Lanotte et al. reported that stromal cell colonies appeared, together with granulocyte-macrophage colonies (CFU-GM), under such conditions (5). They also reported that some foci contained fibroblast-like, endothelial-like and fat cells. They later decided to classify stromal cell colonies into three types; fibroblast-like, mixed fibroblast/macrophage and others (6). However, they did not observe the presence of any capillary-like structures. We also reported that while fibroblastic cells, adipocytes and granulocyte-macrophage colonies appeared in bone marrow cultured in collagen gel, no endothelial cells were evident (10).

Using a Dexter-type liquid culture system (3), adherent cells were constituted with fibroblastic or epithelioid cells, adipocytes, macrophages and endothelial cells. The culture conditions under which capillaries appeared from bone marrow in the col-

lagen gel were observed and examined morphologically.

MATERIALS AND METHODS

Cells. Bone marrow cells were obtained by flushing them from mice femora (C3H/He, 6–8 weeks old) with Fisher's medium by means of a syringe with 23 gauge needle. These cells were carefully dispersed by passing them through a Pasteur pipette several times. The resulting suspension contained about 8×10^6 cells/ml with cell clusters less than 0.2 mm in diameter.

Collagen gel culture. Collagen gel was prepared as previously reported (10). Briefly, 7 volumes of collagen solution (3 mg/ml of type I collagen from porcine tendon) (Cellmatrix Ia, Nitta Gelatin Co. Ltd. Japan), 2 volumes of $5 \times$ Fisher's medium (Gibco) and 1 volume of reconstitution buffer (2.2 g NaHCO_3 in 100 ml of 0.05 N NaOH and 200 mM HEPES) were mixed gently at 4°C . One ml of this solution was then mixed with 1 ml of marrow suspension. This mixture was plated in the centers of four 35 mm Petri dishes (0.5 ml per dish), and allowed to gel at 37°C for 15 min. Each dishes contained about 2×10^6 bone marrow cells. The samples were then overlaid with 2 ml of Fisher's medium containing 20% horse serum, and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C . The medium was not changed during the first two weeks, after which half of the medium was changed weekly.

Electron microscopic observations. Four gels were fixed at 4 weeks of culture with 2.5% glutaraldehyde for 2 hrs at 4°C . They were then post-fixed with 1% OsO_4 for 1 hr, dehydrated through a graded ethanol series and routinely embedded in Epon 812. Ultra-thin sections cut on a Reichert Ultracut E ultramicrotome were stained with uranyl acetate and lead citrate and observed under a JEM 100CX electron microscope.

Fig. 1. Capillary at 1 week after inoculation. (phase contrast, $\times 800$)

Fig. 2. Capillaries (arrows) forming networks among adipocytes having large bright lipid droplets and dark, small, round hemopoietic cells 4 weeks after inoculation. (phase contrast, $\times 300$)

RESULTS

One to two days after inoculation of the bone marrow cells, fibroblastic cells began to migrate from clusters and proliferated. After one week, some of these fibroblastic cells transformed into adipocytes. Hemopoietic cells also proliferated

Fig. 3. Electron micrograph of capillaries 4 weeks after inoculation. Capillary lumina were lined by thin rims of endothelial cells. L: capillary lumen, G: collagen gel ($\times 3,900$)

Fig. 4. High power view of left capillary in Fig. 3. Arrows indicate attachment sites of endothelial cell processes. ($\times 11,000$)

Fig. 5. Pinocytotic vesicles appear preferentially on the luminal side, with a poorly developed basement membrane (arrows) on the abluminal side. ($\times 31,000$)

and formed many clusters.

Small capillary-like structures appeared after one to two weeks. At this time, the capillaries already had lumina with very thin walls (Fig. 1). Four weeks after inoculation, these capillaries contained dilated lumina with thin walls, and formed networks among the adipocytes and hemopoietic cells (Fig. 2). If the number of inoculated bone marrow cells was reduced, both the number of capillaries and hemopoietic cells were reduced as well. Furthermore, if the number of inoculated bone marrow cells was less than 1×10^5 cells/dish, the capillaries and hemopoietic cells completely disappeared and only fibroblastic cells, adipocytes and macrophages remained. If the bone marrow suspensions were redispersed by passing them through a 26 gauze needle until cell clusters disappeared, the capillaries could hardly be observed.

Electron microscopic observations revealed that the capillaries contained relatively large luminal spaces surrounded by endothelial cell thin processes (Figs. 3, 4). Endothelial cells had abundant dilated rough endoplasmic reticulum, a few dense granules and several pinocytotic vesicles on the luminal side (Figs. 4, 5), but Weibel-Palade bodies were not observed. The junctional apparatus between endothelial cells was poorly developed (Fig. 4). Basement membrane was observed only in certain parts of the capillary (Fig. 5). While collagen fibrils could not be found within the capillary lumina, even though they existed outside, cell debris was occasionally observed.

DISCUSSION

The present study demonstrates the appearance of sinusoid-like dilated vessels surrounded by thin endothelial processes from clusters of marrow cells. The quantity inoculated as well as the size of bone marrow cell clusters seem to play important roles in the appearance of these capillaries. Previously we reported that when bone

marrow cells were dispersed with a 26 gauze needle or collagenase digestion, both granulocyte-macrophage colonies and fibroblastic colonies appeared without the presence of endothelial cells (10). This same observation was made by Chertkov *et al.* using a Dexter-type liquid culture system (2). The lot of the horse serum used is another important factor in the appearance of capillaries. It seems that Lanotte *et al.* (6) did not observe capillary structures because they did not use an appropriate serum. The delay in the appearance of vessels during the proliferation of fibroblastic cells and macrophages suggests that these cells play an important role in the appearance of vessels, especially since several fibroblasts and macrophages are known to produce endothelial cell growth factor (4, 7, 14). Xu *et al.* also reported that the growth of endothelial cells appeared to be stimulated by fibroblasts as well as by macrophages in a Dexter-type liquid culture system (16).

Electron microscopically, the capillaries observed in this study contained relatively large luminal spaces surrounded by endothelial thin processes and were generally morphologically different from those reported by other investigators. Capillaries from rat aorta (11, 12), from rat muscle and adipose tissue (9) and from the capillary endothelial cells of bovine adrenal cortex (8) were found to be surrounded by relatively thick walls with the nuclear portion of the endothelial cells protruding into the capillary lumen. We observed no Weibel-Palade bodies in endothelial cells, and Allen has also reported the absence of this body in endothelial cells in mouse marrow cultures (1). These differences suggest that capillaries from cultured bone marrow have characteristics of marrow sinusoids.

REFERENCES

1. ALLEN, T.D. Haemopoietic microenvironments in vitro: ultrastructural aspects. In *Ciba Foundation Symposium 84. "Microenvironments in haemopoietic and lymphoid differentiation."* Pitman, London. pp. 38–67, 1981
2. CHERTKOV, J.L., N.J. DRIZE and O.A. GUREVITCH. Hemopoietic stromal precursors in long-term culture of bone marrow: II. Significance of initial packing for creating a hemopoietic microenvironment and maintaining stromal precursors in the culture. *Exp. Hematol.* **11**, 243–248, 1983
3. DEXTER, T.M., T.D. ALLEN and L.G. LAJTHA. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J. Cell Physiol.* **91**, 335–344, 1977
4. KAN, M., M. KATO and I. YAMANE. Fibroblast products adsorb to the culture substrate and stimulate growth of human umbilical vein endothelial cells. *J. Cell Physiol.* **124**, 125–130, 1985
5. LANOTTE, M., S. SCHOR and T.M. DEXTER. Collagen gels as a matrix for haemopoiesis. *J. Cell Physiol.* **106**, 269–277, 1981
6. LANOTTE, M., D. SCOTT, T.M. DEXTER and T.D. ALLEN. Clonal preadipocyte cell lines with different phenotypes derived from murine marrow stroma: Factors influencing growth and adipogenesis in vitro. *J. Cell Physiol.* **111**, 177–186, 1982
7. MCAUSLAN, B.R., G.N. HANNAN and W. REILLY. Characterisation of an endothelial cell proliferation factor from cultured 3T3 cells. *Exp. Cell Res.* **128**, 95–101, 1980
8. MONTESANO, R., L. ORCI and P. VASSALLI. In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. *J. Cell Biol.* **97**, 1648–1652, 1983
9. MONTESANO, R., P. MOURON and L. ORCI. Vascular outgrowths from tissue explants embedded in fibrin or collagen gels: A simple in vitro model of angiogenesis. *Cell Biol. Int. Rep.* **9**, 869–875, 1985
10. MORI, M., Y. SADAHIRA and M. AWAI. Characteristics of bone marrow fibroblastic colonies (CFU-F) formed in collagen gel. *Exp. Hematol.* **15**, 1115–1120, 1987
11. MORI, M., Y. SADAHIRA, S. KAWASAKI, T. HAYASHI, K. NOTOHARA and M. AWAI. Capillary growth from reversed rat aortic segments cultured in collagen gel. *Acta Pathol. Jpn.* **38**, 1503–1512, 1988

12. NICOSIA, R.F., T.TCHAO and J. LEIGHTON. Histotypic angiogenesis in vitro: Light microscopic, ultrastructural, and radioautographic studies. *In Vitro* **18**, 538–549, 1982
13. NICOSIA, R.F., J.F. MCCORMICK and J. BIELUNAS. The formation of endothelial webbs and channels in plasma clot culture. *Scanning Electron Microscopy*. 793–799, 1984
14. OKABE, T. AND F. TAKAKU. A macrophage factor that stimulates the proliferation of vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **134**, 344–350, 1986
15. RICHARDS, J., G. GUZMAN, M. KONRAD, J. YANG and S. NANDI. Growth of mouse mammary gland end buds cultured in a collagen gel matrix. *Exp. Cell Res.* **141**, 433–443, 1982
16. XU, C.X., J.H. HENDRY, N.G. TESTA and T.D. ALLEN. Stromal colonies from mouse marrow: Characterization of cell types, optimization of plating efficiency and its effect on radiosensitivity. *J. Cell Sci.* **61**, 453–465, 1983

(Received for publication, January 17, 1989 and in revised form, March 6, 1989)