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THE ROLE OF MARROW ARCHITECTURE AND STROMAL CELLS IN THE RECOVERY PROCESS OF APLASTIC MARROW OF LETHALLY IRRADIATED RATS PARABIOSED WITH HEALTHY LITTER MATES

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Abstract

Bone marrow aplasia was induced in rats by whole body lethal irradiation (1,000 rads by x-ray), and rats died of irradiation injury within 7 days.

Correlative studies at light (LM), transmission (TEM) and scanning electron microscopy (SEM) demonstrated swelling of endothelial and reticular cells and hemorrhage due to detachment of sinus endothelial cells on days 1 and 2. With time, structural recovery occurred without hemopoietic recovery. Reticular cells developed small intracytoplasmic lipid droplets on days 3 and 4. This resulted in fatty aplastic marrow within 7 days. On the other hand, in the marrow of irradiated rats parabiosed with healthy mates by aortic anastomosis, hemopoiesis was initiated by adhesion of nucleated blood cells to fine cytoplasmic pseudopods of fat-stored cells on days 1 and 2 after parabiosis. On days 3 to 5, reticular cells with large lipid droplets and fine pseudopods increased, then hemopoietic foci became clear and extensive. On day 8 after parabiosis, the aplastic bone marrow recovered completely both its structure and hemopoietic activity.

Thus, hemopoietic recovery in lethally irradiated marrow begins with recovery of vascular endothelial cells, re-establishment of sinusoidal structure, and morphological and functional recoveries of reticular cells from fat-storage cells by releasing intracytoplasmic lipid droplets. Marrow stromal cells, namely reticular, fat-storage and fibroblastoid cells, share a common cellular origin, and regain their structure and function when fat-storage cells and fibroid cells are placed in contact with hemopoietic precursor cells.

KEY WORDS: Irradiation, Marrow stromal cells, Hemopoietic recovery, Rat parabiosis, Electron microscopy.

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Introduction

Aplastic anemia is attributed to either depletion of bone marrow stem cells (1,13,23) or to dysfunction of bone marrow stromal cells (1,33,34) or their combination (5-8,36,41). There have been many studies on hemopoietic stem cells and their differentiation in vitro (3,13,23,27,43) and in vivo. (15,26,28,29,30,35,42). On the other hand, hemopoietic inductive microenvironment (HIM), proposed by Trentin and Wolf (36,40,41), mainly compoded of reticular cells, macrophages, fat cells and cells of blood vessels (2,9,10,12,14,20,21,25,31,32, 37,38), has been recognized in vitro (10,14,20, 22), and in vivo (2,5-9,12,25,27,31,32,37-39). However, details of cellular activities and their function in hemopoiesis have not been fully elucidated.

Recently, marrow stromal cell lines, derived from marrow reticular cells with morphological appearance of fibroblastic cells or fat cells, were established in vitro (14,20). These cells play an important role in inducing hemopoietic cell differentiation and proliferation by releasing factors of hemopoietic cell differentiation (10,14,20, 22,31,32). Only a few studies are available on morphological alteration of marrow stromal cells, their fatty transformation, and their reversion to hemopoietic marrow in vivo (18,19,31,32).

Ultrastructural studies of bone marrow have highlighted a close association of cytoplasmic processes of stromal cells in hemopoietic cell differentiation and proliferation. However, there are only a few coordinated studies on light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (12, 21,25,31,32,37). We have attempted to combine these three methodologies to overcome previous difficulties in examining hematopoietic organs with only two-dimensional images.

This paper reports on LM, TEM, and SEM findings of stroma and sinal vasculature of marrow undergoing fatty transformation and aplasia induced by lethal irradiation. We have further studied the recovery process in this system after parabiosis in rats.

Materials and Methods

Male Wistar rats weighing 300 to 320g were used. The methods have been described earlier (11, 16,17). Briefly, the bone marrows of untreated

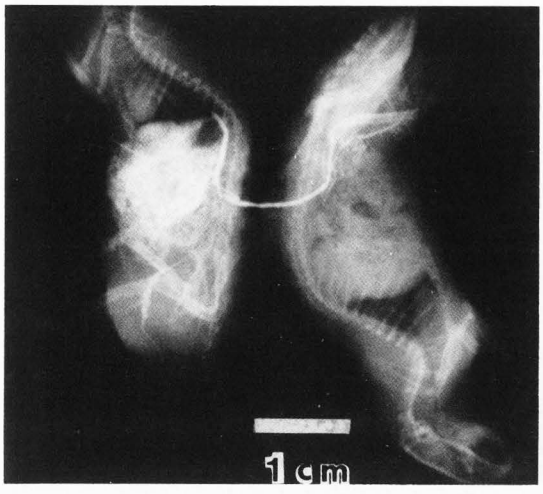


Figure 1. High-speed photograph of parabiosed rats. Cross-circulation of blood between rats, shown by injected radiopaque substance (76% sodium iothalamate).

normal and lethally-irradiated rats with or without serum transfusion were used as controls. The marrow of a rat parabiosed with a normal littermate by aortic anastomosis (Fig. 1) on day 3 of post-irradiation was also studied.

Marrow was prepared with perfusion-fixation with 2% glutaraldehyde in phosphate buffer (pH 7.2, 300ml). For LM, tissue sections were stained with hematoxyline-eosin, silver impregnation, azan and elastica-van Gieson. Ultrathin and semithin sections from epon embedded blocks were used for TEM. For SEM, specimens were prepared by Murakami's method (24). Ultrathin sections were cut with Ultracut E (Reicher-Jung), dipped in a mesh (Veco-150) covered with colloidine membrane, doubly stained with uranyl acetate and lead citrate, and examined in a JEM-100CX operated at 80kV.

Results

LM observations

One to 2 days after irradiation, severe hemorrhage and necrosis of hemopoietic cells were noted. On day 3, the marrow was empty (Fig. 2B) and there was a tendency to a fatty involution. These rats died within 7 days of aplastic fatty marrows (Fig. 2C) irrespective of whether serum was transfused or not (16,17). By contrast, irradiated rats parabiosed with healthy litter-mates, irradiation was also followed by hemorrhage and necrosis (Fig. 2D), but by day 3, hemopoietic recovery was evident (Fig. 2E). Hemopoietic activity reached the same level as controls (Fig. 2A) on day 8 (Fig. 2F).

Observations on special-stained sections revealed fibrosis in the irradiated bone marrows (16). Fibrosis persisted despite complete recovery of hemopoiesis on day 8 after parabiosis (16).

Postirradiation hemorrhage and necrosis of hemopoietic cells were also observed in semithin sections stained with toluidine-blue. Macrophages, reticular and sinal endothelial cells were easily distinguished from each other (Fig. 3A). On day 3, hemorrhage was still present and reticular cells with large and small lipid droplets were noticed (Fig. 3B).

Figure 2(A-F). Light microscopic observations of rat bone marrow. H.E.. A : Non-irradiated. B : Three days after irradiation, showing disappearance of hemopoietic cells and progressive fatty change of stroma. Hemorrhage still remained. Irradiated rats with fatty marrow were conjugated with healthy litter mates. C : Seven days after irradiation, showing complete fatty aplastic marrow. Rats die within 7 days. No hemopoietic recovery was seen in the rats with large volume of serum transfusion. D : One day after parabiosis. Nucleated blood cells from healthy partners are scattered with a tendency of cluster formation. E : Four days after parabiosis, showing extensive hemopoietic distribution. F : Eight days after parabiosis, showing complete recovery of hemopoiesis. Hemopoietic cell distribution is the same as normal bone marrow(A). ➡

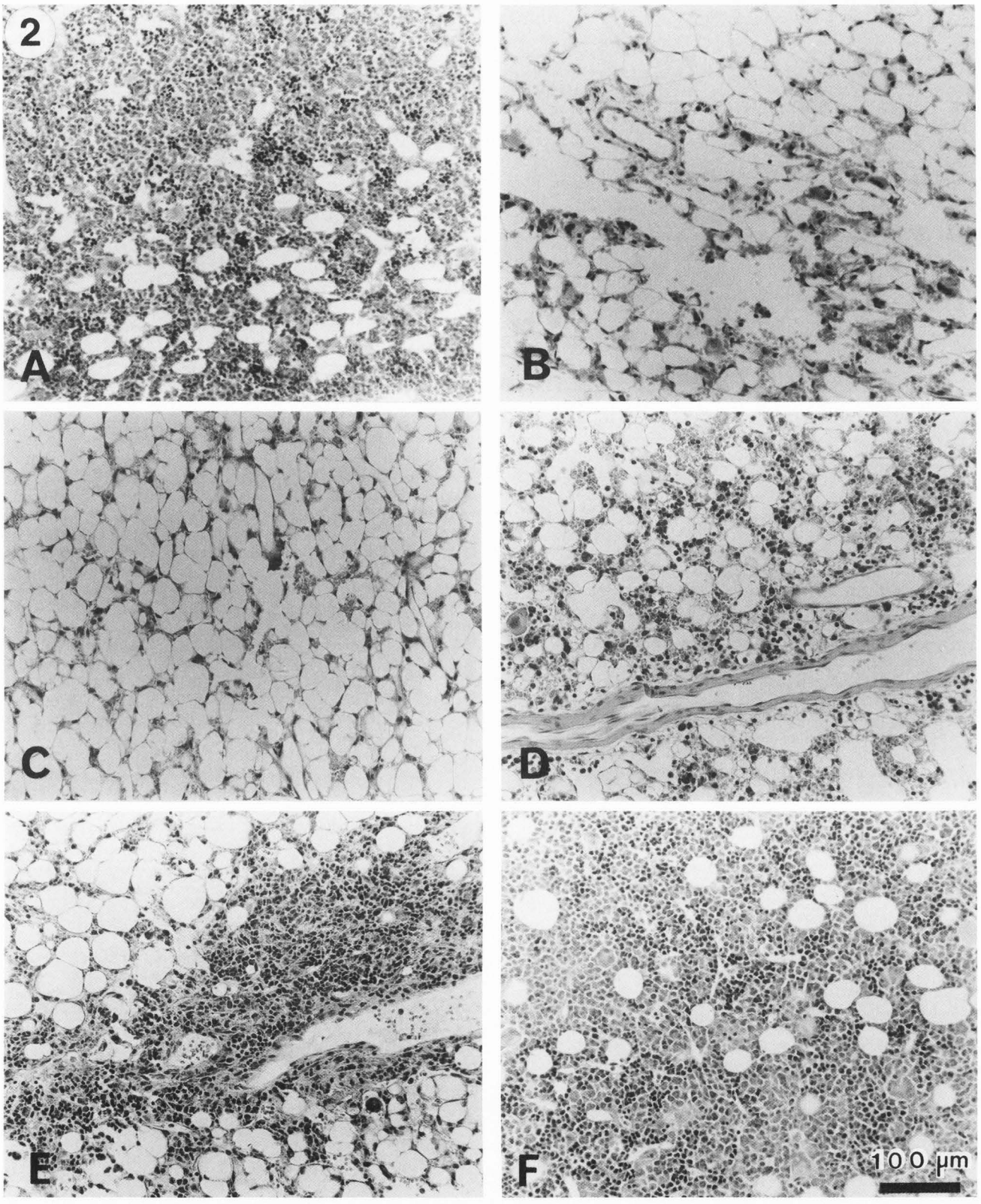
Thereafter, fatty change progressed with an increase in the number of reticular cells having large lipid droplets, resulting in complete fatty marrow (Fig. 3C). Fibroblastoid reticular cells with fine pseudopods and small intracytoplasmic droplets were scattered around blood vessels and in intersinusoidal spaces (Fig. 3C). An important finding observed in semithin sections was the developing process of fat-storage and fibroblastic cells from reticular cells, although morphological differences between inter- and peri-sinusoidal reticular cells were almost impossible.

On day 1 after parabiosis, hemorrhage remained and sinusoidal structure was most indistinct. However, by day 2 sinusoidal structure was more recognizable and a few nucleated blood cell clusters were noted (Fig. 3D). Thereafter, the recovery of sinusoidal structure proceeded and hemopoiesis resumed. These findings became prominent on day 4 after parabiosis (Fig. 3E). Hemopoietic foci enlarged in parallel with the decrease in fat component. Bone marrow structure and hemopoietic activity reached about the same level as in the controls on day 8 (Fig. 3F).

TEM observations

In the marrow of healthy rats, reticular cells were found occasionally, and their cytoplasmic pseudopods were hardly recognized because of a high density of hemopoietic cells (Fig. 4A). Macrophages were also rarely seen.

On day 1 of post irradiation, not only swelling, degeneration and necrosis of hemopoietic cells, but also diffuse hemorrhage was observed. This was due to detachment of sinus endothelial cells. As hemopoietic cells disappeared, reticular cells, macrophages and their cytoplasmic pseudopods were easily identified, especially in reticular cells containing lipid droplets. Macrophages phagocytized large cellular debris and many lysosome granules of variable size (Fig. 4B). This picture intensified on day 3 of post irradiation. Hemopoietic cells totally disappeared, and reticular cells including fibroblastic cells with intracytoplasmic lipid droplets and fine cytoplasmic pseudopods were easily identified (Fig. 4C). On days 5 to 7 of post irradiation, fatty transformation was found in the marrow with reticular cells containing large lipid droplets, and stromal collagen fibers increased (Fig. 4D). A difference in the marrow structure of parabiosed rats was the presence of nucleated cells on day 1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after parabiosis. These cells adhered closely to fine cytoplasmic pseudopods of lipid containing reticular cells and also macrophages (Fig. 4E). Small hemopoietic foci were observed on day 2; they increased in number and size and increasingly interdigitated with fine cytoplasmic processes of reticular cells. On day 4, parallel with progressive activity of hemopoiesis, lipid containing reticular cells were rarely seen. Spindle-shaped fibroblastoid cells, with numerous pseudopods and few small intracytoplasmic lipid droplets, were occasionally encountered (Fig. 4F). On day 8, hemopoietic distribution was almost normal, and because of dense distribution of hemopoietic cells, reticular cells and macrophages were difficult to observe. They extended their fine cytoplasmic pseudopods into hemopoietic cells (Fig. 4G).

SEM Observations

In control rats, the surface of sinus endothelial and stromal cells was smooth. The outline of sinusoidal structure was not clear because of a dense distribution of hemopoietic cells (Fig. 5A).

In lethally irradiated rats, on the first 2 days of post-irradiation, severe surface irregularities and swelling of vascular endothelial cells were seen (Fig. 5B). By day 3, however, these changes improved (Fig. 5C). Further improvement was noted on day 4, as the sinusoidal and finely reticulated structures of cytoplasmic pseudopods became clear. Of particular interest were changes in cells with pseudopods. On day 1 of post-irradiation, pseudopods were thick, coarse and short (Fig. 5B). On day 2, these cytoplasmic processes became relatively thin, and stretched in all directions. On day 3, parallel to the increase in numbers of fat-storage cells, these processes became thinner, increased in numbers and interdigitated to make a coarse reticulated structure (Fig. 5C). On days 4 to 5 of post-irradiation, the reticular nets of fine fibrous pseudopods was increased. In the fatty marrow on day 7, the interdigitated fine reticular processes, which were considered to be cytoplasmic pseudopods of fat-storage reticulum cells, formed reticular meshes (Fig. 5D).

In parabiosed animals, stromal cytoplasmic reticular processes were still coarse on day 1. By day 2 after parabiosis, a notable recovery in sinusoidal endothelium was found, and the intricated reticular structures of fine cytoplasmic pseudopods were observed in intersinusoidal spaces (Fig. 5E). With the increased number of pseudopods in reticular cells, these processes became thinner and hemopoietic foci clear (Fig. 5F). Following an increase in numbers and sizes of hemopoietic colonies, on day 8 of parabiosis, the bone marrow structure and hemopoietic recovery reached the same state (Fig. 5G) as healthy control rats (Fig. 5A).

Discussion

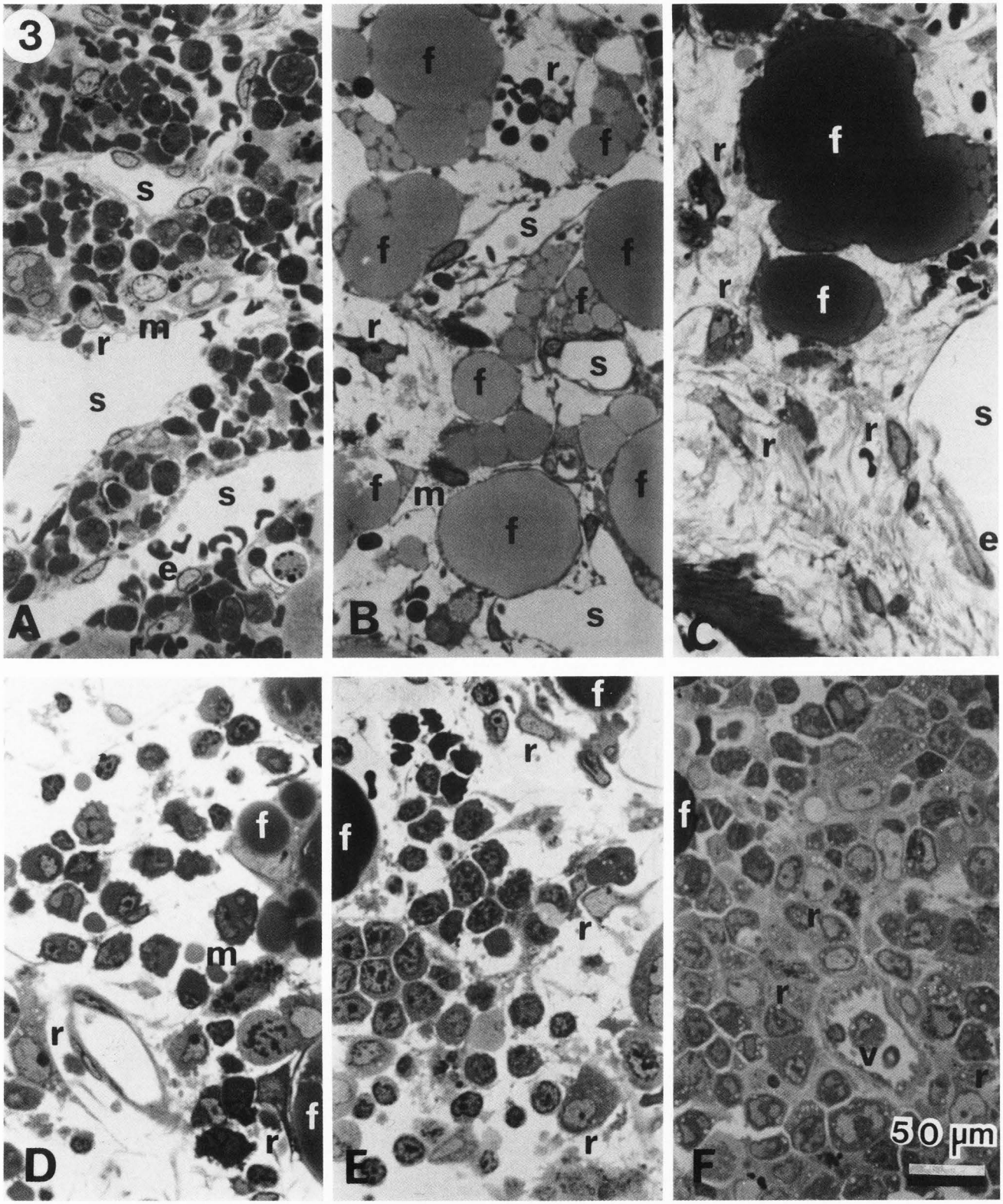
The concept of HIM (36,40,41) has been confirmed with studies in vitro (10,14,20,22) and in vivo (2,5-9,12,25,27,31,32,37-39). The HIM is composed of the cells such as inter- and peri-sinusoidal reticular cells, fat cells, fibroblastoid cells, macrophages and the cells of vasculatures (2,9,10,12,14,20,21,25,31,32,38,39). However, the details of inter-relationships of these cells including architecture of stroma and their relationships to hemopoiesis are not fully elucidated (31,32,38,). The difficulties in studying marrow stromal cells in vivo depends mainly upon highly dense distribution of hemopoietic cells in the bone marrow of normal animals (37,38). A lethal dose of irradiation destroys hemopoietic cells but not stromal cells. (4,18,19). Therefore, we were able to observe how fatty aplastic marrow develops from lethally-irradiated marrow stromal cells after disappearance of hemopoietic cells (Figs. 2B,2C,3A-C,5A-D).

Figures 3(A-F). Observations in semithin sections. Toluidine blue. e; endothelial cell, f; fat storage cell, m; macrophage, r; reticular cell, s; sinus, v; vein. A : One day after irradiation, showing hemopoietic cell degeneration and hemorrhage. Sinal endothelial cells, macrophages and reticular cells are distinguished from each other. B : Three days after irradiation, showing reticular cells with lipid droplets in cytoplasm. Note the lipid droplets vary in size. C : Seven days after parabiosis, showing reticular cells with tiny lipid droplets, and spindle-shaped fibroblastoid cells in fibrous matrix. D : Two days after parabiosis, showing hemopoietic cell clusters and reticular cells with or without lipid droplets. Reticular cells with or without a few lipid droplets remarkably increased. F : Eight days after parabiosis, showing normal distribution of hemopoietic and original shaped reticular cells.

In lethally-irradiated rats, hemorrhage occurs due to injury of sinal endothelial cell membranes and detachment of their junctions (Figs. 2B,3A,4B). Following withdrawal of the hemorrhage, reticular cells accumulate lipid droplets in the cytoplasm, and develop into fat-storage cells (Fig. 3B) or transform to spindle-shaped fibroblastoid cells, which have a few intracytoplasmic small lipid droplets and an ability of producing collagen fibers (Fig. 4D).

Therefore, fibroblastoid cells can induce fibrous marrow, whereas fat-storage cells produce fatty marrow; fibrous and fatty marrow is known to be induced by irradiation (18,19). Reticular fibroblastoid and fat-storage cells may arise from the same type of original cells (2,25,31,32,39). Peri- and intra-sinusoidal reticular cells are morphologically difficult to distinguish; they are only recognized by their localization in peri- or intersinusoidal areas (Figs. 4A,4B). Macrophages are easily identified from reticular cells by intracytoplasmic organellae and phagocytic activity (Fig. 4B).

Lethally-irradiated animals can recover their hemopoietic activity by transplantation of bone marrow cells (5-8,40,41) or parabiosis with normal partners (26,28,29). However, the mechanism of the recovery process and the role of stromal cells in this recovery are not clear. Hemopoietic recovery begins with the recovery of stromal architectures (Figs. 4B-G), including morphological and functional alterations of fat-storage cells or fibroblastoid cells by releasing intracytoplasmic lipid droplets, and loss of ability of collagen production. This results in the original form of reticular cells (Fig. 4G). The fatty marrow after parabiosis (Figs. 2B,2C,3B,3E) suggests: (1) that fat-storage cells are able to induce hemopoietic differentiation when they are in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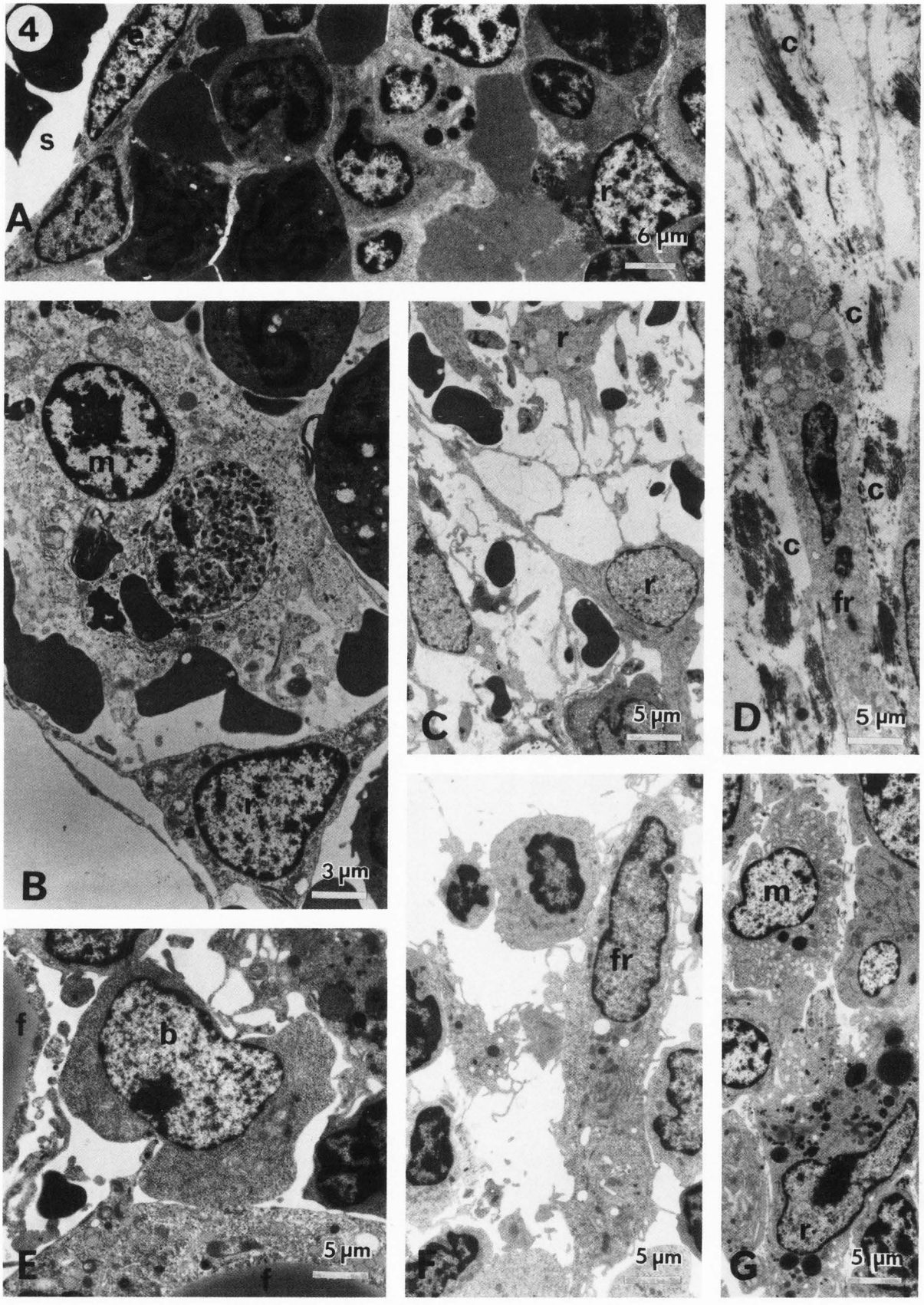
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contact with hemopoietic precursors, and (2) that they regain their morphological structures and functions when they lose cytoplasmic lipids (Figs. 4E-G). The morphological and functional recovery can also be explained by cell-to-cell interaction between these cells and hemopoietic cells. Derivation of hemopoietic precursors was demonstrated earlier (26,28,29). They are trapped by fine cytoplasmic pseudopods of reticular cells and macrophages, and differentiated (Figs. 3D-F,4E-G).

In conclusion, (1) recovery of hemopoiesis in lethally-irradiated rats parabiosed with a normal littermate requires re-establishment of sinal vasculature including recovery of fine pseudopods of reticular cells and macrophages. (2) Circulating hemopoietic precursors from normal partners are trapped by pseudopods of fat-storage or fibroblastoid cells. And (3) trapped hemopoietic precursors stimulate these stromal cells to regain their original shape and function as reticular cells to induce hemopoietic cell differentiation.

Acknowledgements

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Figure 4(A-G). TEM observations. b; blastic cell, c; collagen fibers, e; endothelial cell, f; fat-storage cell, fr; fibroblastic cell, m; macrophage, r; reticular cell, s; sinus. A : Non-irradiated bone marrow, showing peri- and inter-sinal reticular cells. These cells are morphologically difficult to be recognized, and only distinguished by the presence in peri- or inter-sinusoidal areas. B : One day after irradiation, showing a macrophage and a reticular cell with a few small lipid droplets. Macrophage is easily distinguished by phagocytic activity and phagosomes. Red cells are extravasated due to detachment of sinal endothelial cells. C : Three days after irradiation, showing reticular cells with small lipid droplets and flagellated pseudopods extending in all directions. These pseudopods are comparable with the findings shown in SEM (Fig. 5C, 5D). The sinal structures are obscure due to detached endothelial cells and extravasated red cells. D : Seven days after irradiation, showing a fibroblastoid reticular cell with small lipid droplets. Large amounts of collagen fibers around a cell might be produced by the cell. Pseudopods are scanty in this fibroid cell with collagen production. E : One day after parabiosis, showing a blastic cell closely adhered to pseudopods of reticular cells with large lipid droplets (fat-storage cells). F : Four days after parabiosis, showing a fibroblastoid reticular cell with a number of pseudopods. Flagellated pseudopods are closely adhered to hemopoietic cells. This type of spindle-shaped reticular cell does not produce collagens (refer Fig, 4D). G : Eight days after parabiosis, showing a reticular cell with small lipid droplets and a macrophage with extremely intricate pseudopods.

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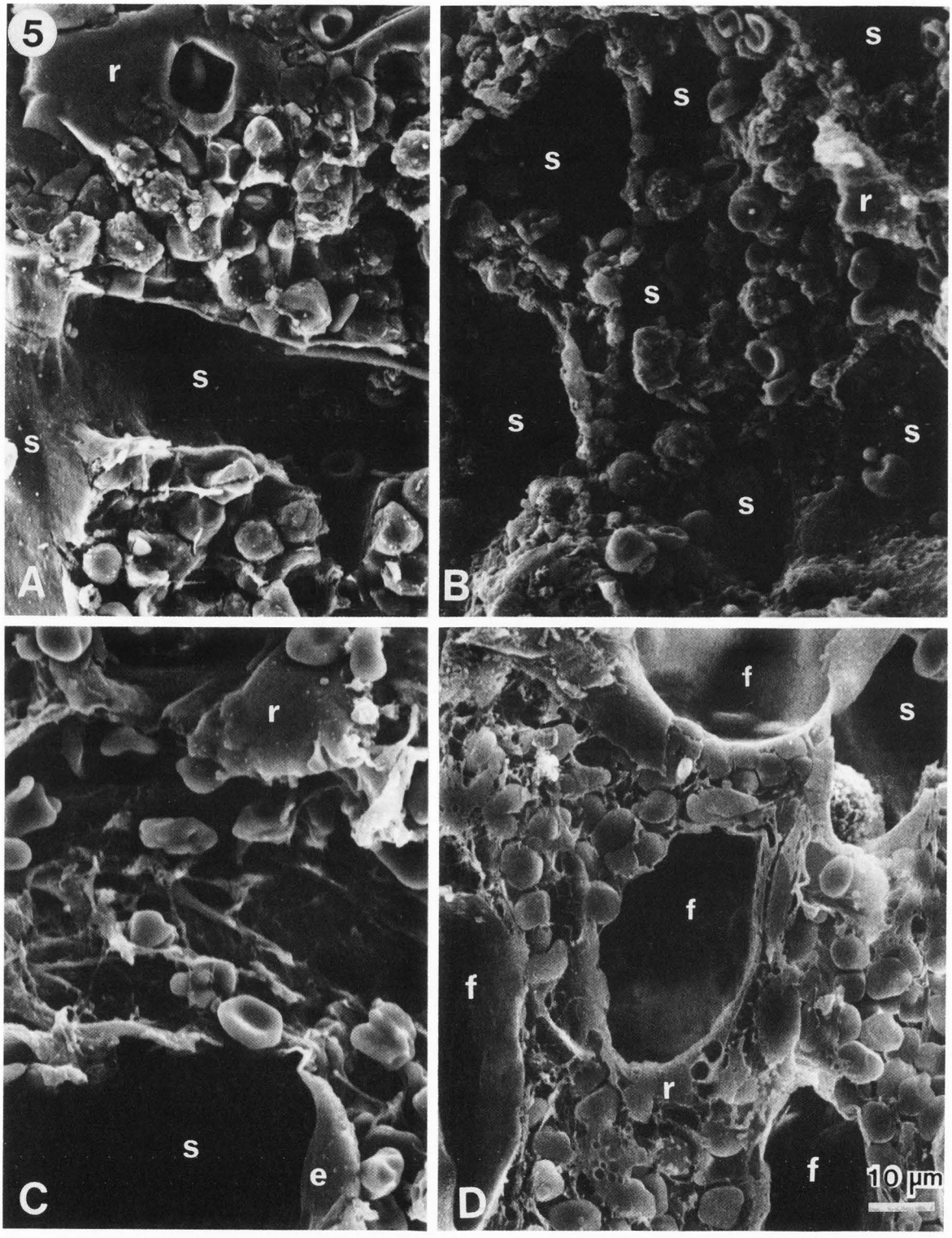
See pages 1496 and 1497 for Figure 5.

Figure 5(A-G). SEM observations. e; endothelial cell, f; fat-storage cell, r; reticular cell, s; sinus. A: Non-treated rat bone marrow. B : One day after irradiation, showing surface irregularities and swelling of sinal endothelial cells. Fine pseudopods of reticular cells diminished, suggesting destruction or fragmentation of the pseudopods. C : Three days after irradiation, showing recovery of surface irregularities and cytoplasmic swelling of sinal endothelial cells. Relatively thick reticular structures suggests recovery of cytoplasmic pseudopods of reticular cells (refer Fig. 4C). D : Seven days after irradiation. Note intricate reticular processes of fat-storage cells. E : Two days after parabiosis, showing intricate reticular structures of fine cytoplasmic pseudopods. Cytoplasmic lipid droplets are smaller than those of bone marrow in irradiated controls, suggesting loss of cytoplasmic lipid droplets after parabiosis. F : Four days after parabiosis. The cytoplasmic processes become thinner. Note pseudopods of reticular cells extend to all directions. G : Eight days after parabiosis, showing the structure and hemopoietic cell distribution to be the same as untreated rat bone marrow.



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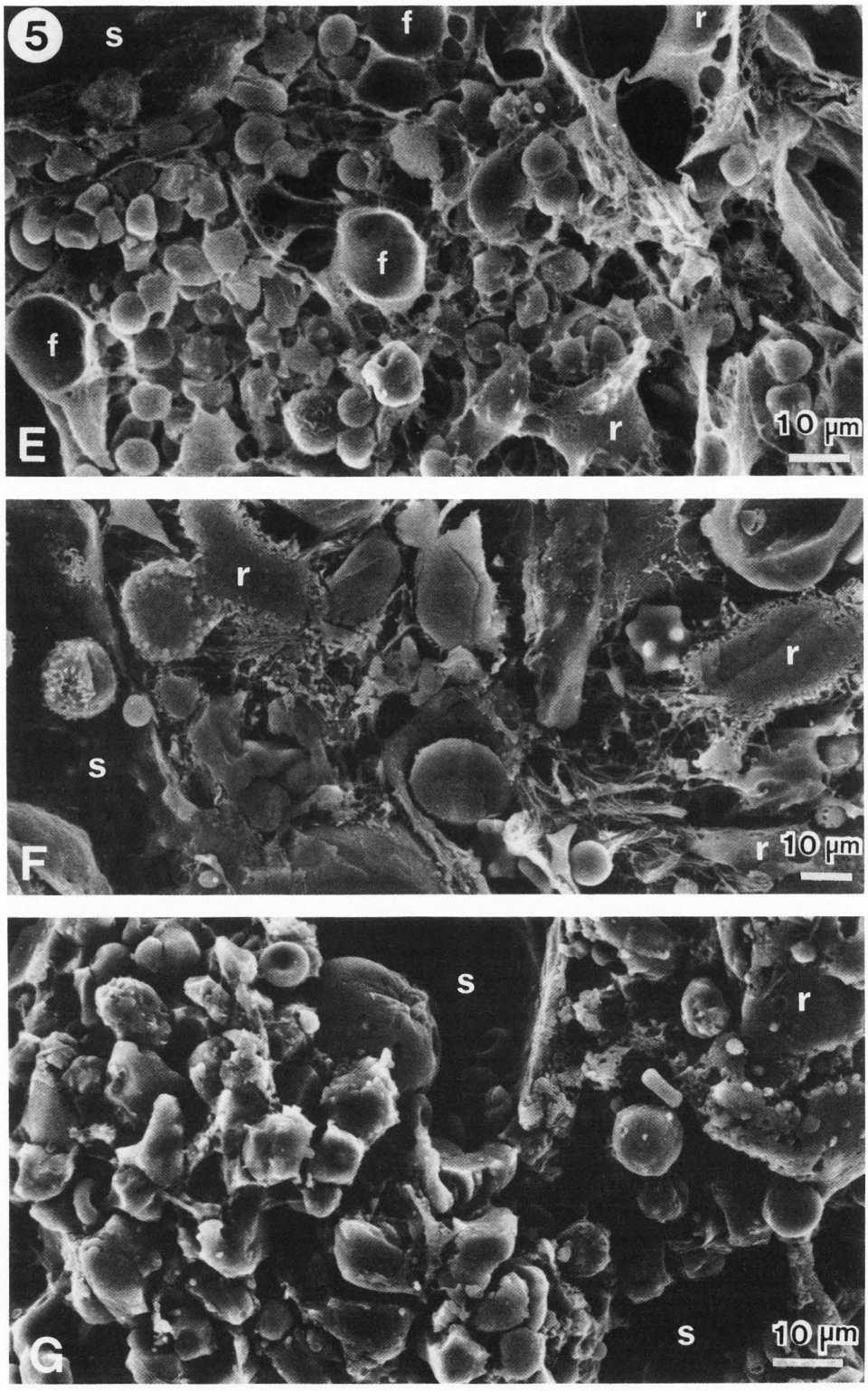
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Discussion with Reviewers

M. Tavassoli: What is the relationship of reticular cells, fibroblastoid cells and fat-storage cells? Are they one cell type in different functional stages or 3 different cell types?
Authors: We think that they are originally one-cell type with different functional stages. As shown in the text, marrow reticular cells, originally having no or a few intracytoplasmic lipid droplets and also having no ability of collagen production, became fat-storage cells resembling adipocytes or fibroblastoid cells with collagen production. These cells have returned to the original reticular cells after parabiosis.

M. Tavassoli: How do the pseudopods recognize and trap hemopoietic precursors? Is there a receptor-ligand interaction involved? This is particularly pertinent since recent evidence in lymphocyte homing points out toward a membrane lectin interacting with membrane glycoprotein and it is possible that a similar mechanism may be involved in the homing of hemopoietic precursors. Moreover, similar pseudopods (microvilli) have been reported on the luminal surface of marrow endothelium, and are thought to be responsible for trapping of stem cells (Soda R, Tavassoli M: J. Ultrastruct. Res. 84:299-310, 1983).
Authors: Based on the morphological findings that pseudopods of reticular cells closely adhered to hemopoietic cells in the initial stage of hemopoietic recovery, we speculated that hemopoietic precursors were trapped by pseudopods of reticular cells. However, at present, we have no evidence concerning receptors, such as lectin-related, of reticular cells, macrophages and sinal endothelial cells. As shown by Soda and Tavassoli (1983, see above), their receptor theory will be reasonable, because histoanatomically and chronologically hemopoietic precursors in blood need to traverse vascular endothelial cells before adhering to reticulum cells. Endothelial cells, therefore, may select and trap hemopoietic cells. However, at the moment, we have no evidence to prove the above facts. This needs further investigation.