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Studies on The Adherent Layer in Long-Term Bone Marrow Culture of Human Fetuses

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ABSTRACT

Long-term bone marrow cultures of human fetuses ranging from 20 weeks to 28 weeks were established. The following methods were used to study the adherent layer: 1) Giemsa stain, Sudan IV stain, and Gomori silver impregnation. 2) immunocytochemical staining. 3) phagocytosis experiment with *C. albicans*. 4) transmission and scanning electron microscopy. 5) ultrastructural cytidine monophosphatase (CMPase) and myeloperoxidase (MPO) reaction.

The results indicated that fibroblasts and macrophages (MØs) were principal cell components of the adherent layer. The existence of adipocytes and endothelial cells could not be proved. Fibroblasts were characterized by a large amount of rough endoplasmic reticulum and formation of reticular fibers and fibronectin (FN). Most MØs were capable of engulfing *C. albicans*. The salient morphologic features of fetal MØs was the presence of large numbers of clear vacuole with positive CMPase reactivity. Extracellular matrix (ECM) were composed of FN, proteoglycan and reticular fibers. Together with the fibroblasts and MØs, they composed the hematopoietic inductive microenvironment in vitro, in which the proliferation and maturation of myelomonocytic cells were sustained.

KEY WORDS

Human fetus, Long-term marrow culture, Stromal cells, Extracellular matrix, Hematopoietic inductive microenvironment.

INTRODUCTION

The effects of hematopoietic inductive microenvironment (HIM) upon hematopoiesis have been fully proved. In 1977, Dexter et al [1] developed long-term culture of murine bone marrow, which sustained growth of hematopoietic stem cells in vitro. The structure and function of the adherent layer in this system are analogous to those of HIM. Since then the studies on long-term bone marrow culture (LTBMC) have received much attention [2, 3,4]. However, there is no general agreement as to the components of the adherent layer. We studied the LTBMC of human fetuses, in the hope of throwing light on this problem from an ontogenetic approach.

MATERIALS AND METHODS

Bone marrow culture

Eight normal fetuses from 20 to 28 weeks obtained by induced abortion were studied. Bone marrow cultures were prepared using the method recommended by Gartner and Kaplan [2] with modification. Briefly, femoral marrow was extruded into RPMI 1640 medium(GIBCO) with 10% fetal bovine serum. Nucleated cells were isolated and suspended in IMDM (GIBCO) plus 10% fetal bovine serum, 10% house serum and $5X10^{-7}$ mol/L hydrocortisone. They were plated at a density of $5X10^5$ / ml on six-well culture dishes with glass coverslips. Cultures were incubated at 33°C in air containing 5% CO^2 and fed weekly by replacing half of the medium with fresh medium. In the mean while, some coverslips were removed for following experiments.

Cytological and immunocytochemical stains

Cells and extracellular materials were studied after Giemsa stain, Sudan VI stain and Gomori silver impregnation. Immunocytochemical stains were processed according to the method of Shi [5], with rabbit antiserum specific for FN (produced by Beijing

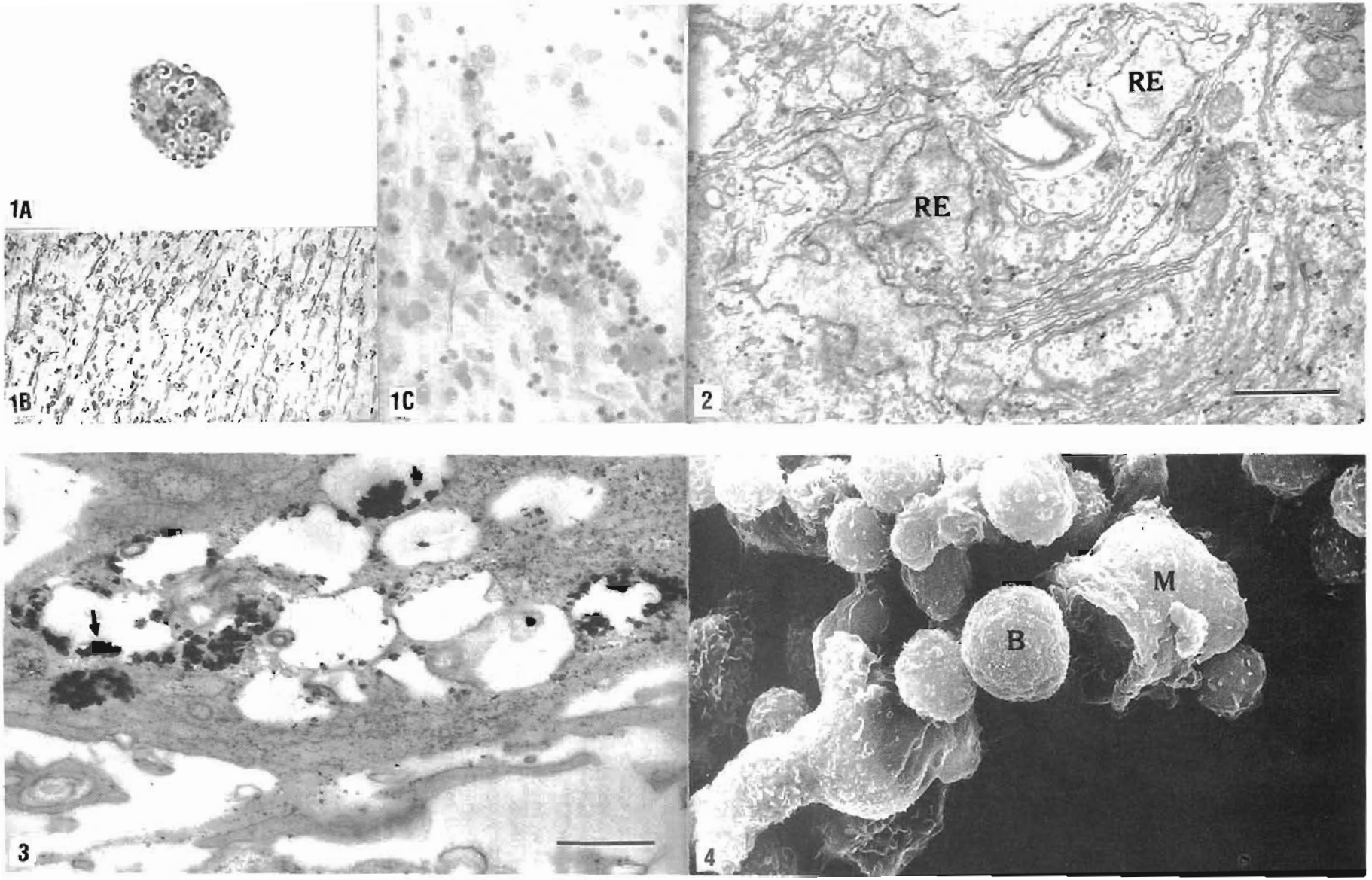


Fig. 1A Phagocytosis experiment showing engulfed *C. albicans* in the cytoplasm of MØ. Mag 400X.

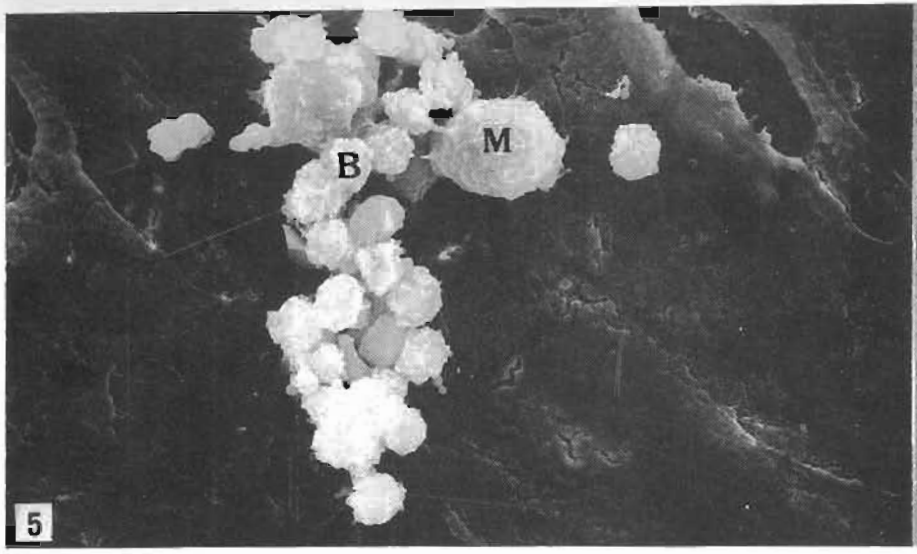
Fig. 1B Gomori silver stain revealing reticular fibers. Mag. 100X.

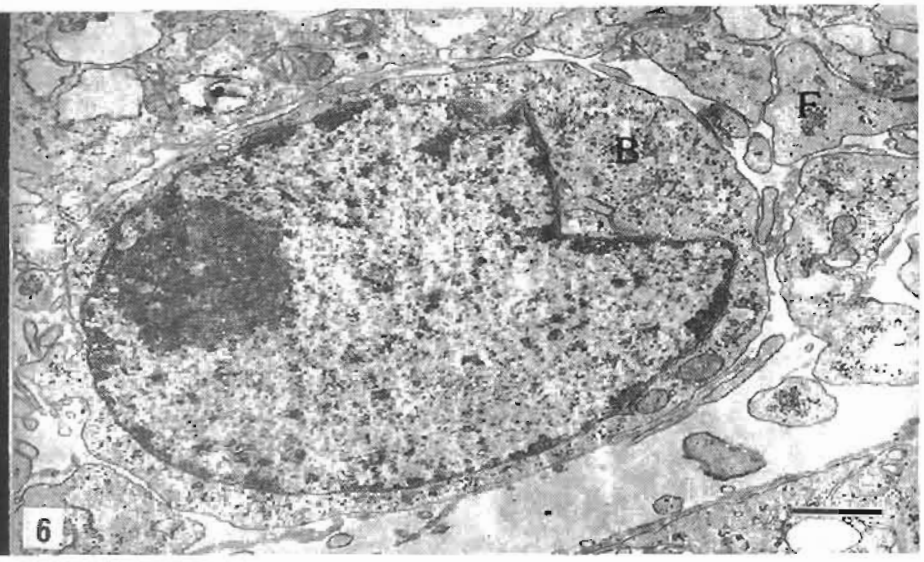
Fig. 1C Light microscopy showing a confluent layer of fibroblasts with a superimposed hematopoietic island. Giemsa stain. Mag. 200X.

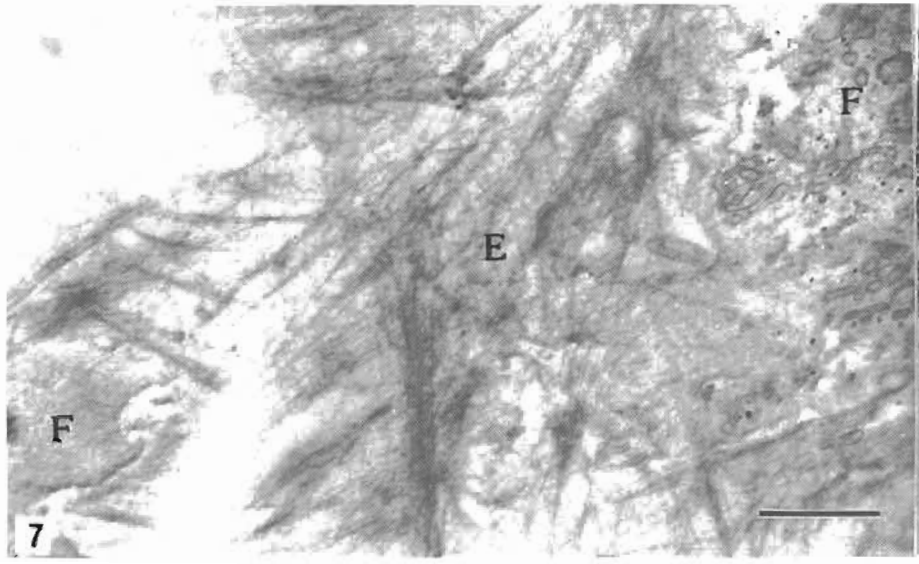
Fig. 2 Part of fibroblast showing a large number RER cisternae, some of which (RE) distended by flocculent material. Bar = 0.5um.

Fig. 3 CMPase reaction product (arrow) in cytoplasmic vacuoles of MØs. Bar = 0.5um.

Fig. 4 SEM revealing crater-like depression and ruffles on the MØ (M), also ridges and microvilli on the round hematopoietic cells (B). Mag. 2600X.







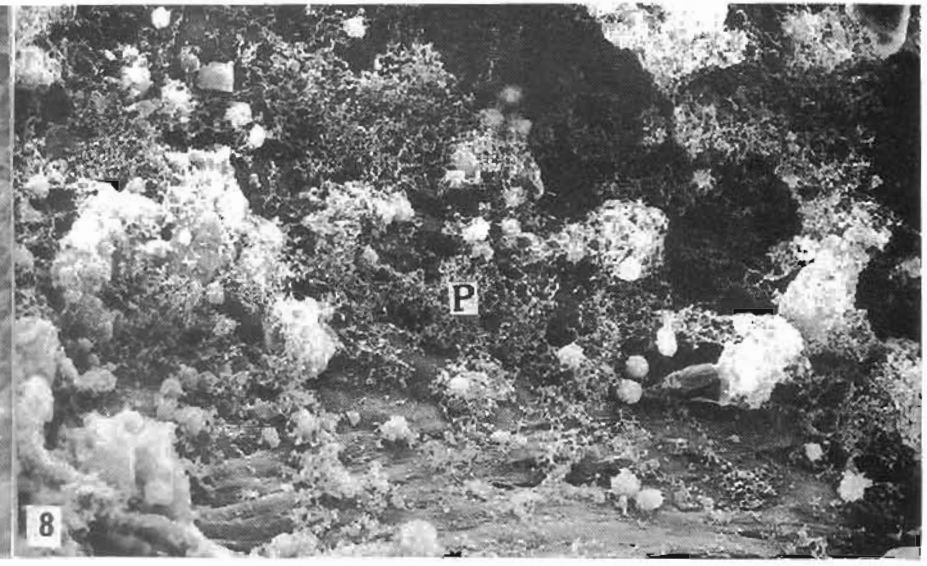


Fig. 5 SEM showing a hematopoietic island containing hematopoietic cells (B) and MØs (M) lying on a confluent layer of fibroblasts. Mag. 1300X

Fig. 6 Primitive hematopoietic cell (B) with a large nucleus, prominent nucleolus and small amounts of organelles, surrounded by cytoplasmic processes of fibroblasts (F). Bar = 1um.

Fig. 7 Extracellular fibrillar (E) material on the surface of fibroblasts (F). Bar = 0.8um.

Fig. 8 A mat-like layer (P) of proteoglycan surrounding hematopoietic islands and cells on the surface of fibroblast layer (ruthenium red staining). Mag. 540X.

Mediacal Universiy), monoclonal antibodies specific for VIIIR: Ag, platelet glycoprotein (GP) Ib, GPIIb/IIIa respectively (provided by Suzhou Medical College), and Histostain-SP kits (Zymed Laboratory).

Transmission and scanning electron microscopy

Some coverslips of LTBMC were fixed in 3% glutaraldehyde and 1% osmium tetroxide respectively, then dehydrated in graded concentrations of ethanol and embedded in Epon in situ. Cultured cells on other coverslips were gently removed by scraping with a rubber policeman, the pellets were processed as above. Ultrathin sections were stained in uranyl acetate and lead citrate and observed under Hu-12A electron microscope. For scanning electron microscopy (SEM), coverslips were fixed as above, dried in a critical point drier, and coated with gold-palladium. Micrographs were taken with a JSM-35CF scanning electron microscope. Besides, cultures were stained with ruthenium red according to the method described by Wright [6]. Ultrastructural cytidine monophosphatase (CMPase) reaction was processed using Tang's method [7], and myeloperoxidase (MPO) reaction processed according to that of Graham and Karnovsky [8].

Phagocytosis experiment

C. albicans culture was rinsed with normal saline and inactivated in 70° C for 1 hour, and then suspended in IMDM. Cultured cells and *C. albicans* were mixed at the rate of 1:10, and incubated in dishes containing glass coverslips in 37° C for 1 hour. Some coverslips were observed with light microscope after Giemsa staining. Others were prepared for transmission electron microscopy (TEM).

RESULTS

Light microscopy

The following cell types were observed in the adherent layer. Fibroblast were polygonal or spindle shaped with thin and long cytoplasmic processes, and prominent nucleolus in their oval nucleus. The cytoplasm was rich and appeared a gray blue or reddish color after Giemsa staining. During the early days of culture, frobroblasts distributed individually, and began to form a confluent layer at two weeks. Macrophage were round or oval shaped and different in sizes ranging from 10 to 30μm in diameter, with round nucleus but indistinct nucleolus. Their cytoplasm didn't stained evenly, sometimes with a granular appearance. Phagocytosis experiment showed most MØs were capable of engulfing *C. albicans* (Fig. 1A). Both fat cell and endothelial cell couldn't be discerned, only some MØs stained faintly with Sudan IV, and those small and round cells stained positively with anti-VIIIR:Ag monoclonal antibody were considered to be primitive hematopoietic cells.

The cytoplasm of both fibroblasts and MØs stained positively with anti-FN antiserum after 1 week of culture. Extracellular fibrillar materials connecting fibroblasts were also labelled after 2 weeks. At the same time, reticular fibers began to appear and increased with time (Fig. 1B).

A large number hematopoietic cells existed at 1 week of culture. But hematopoietic islands appeared only after 2 weeks (Fig. 1C), which consisted predominantly of ganulocytes and monocytes. But some primitive hematopoietic cells could react with anti-GPIb and anti-GPIIb/IIIa monoclonal antibodies.

Electron microscopy

Fibroblasts had an euchromatic nucleus, and contained many mitochondria, developing Golgi complex and some cisternae of rough-surfaced endoplasmic reticulum (RER) in their cytoplasm. During the early days of culture the amount of RER increased with time, whose cisternae might be distended with flocculent material of low electron density (Fig. 2). Some cisternae of Golgi complex were also distended, accompanying many vesicles everywhere in cytoplasm. SEM showed these cells were flattened and began to coalesce into a confluent layer at 2 weeks. The nuclei of MØs were somewhat heterochromatic. Their cytoplasm contained a large number of lysosome, phagosome, and vacuole, all of them were bounded by a limiting membrane, with flocculent or clear contents showing positive CMPase reactivity (Fig. 3). MPO activity was absent in MØs. Phagocytosis experiment showed *C. albicans* in their cytoplasm. SEM revealed that MØs had many ridges, ruffles and even crater-like depression (Fig. 4), indicating high level of surface activity. In the hematopoietic islands some MØs contacted with hematopoietic cells via microvilli. The latter cells were round shaped, with some ridges or broad ruffles, or only a few microvilli on their surface (Fig. 5,). Positive MPO products could be seen in perinuclear cisternae, endoplasmic reticulum and granules of most immature and maturing hematopoietic cells, it was indicated that

the principal cell types belong to myelomonocytic line. Fibroblasts were also on intimate associations with hematopoietic cells which were often surrounded by their cytoplasmic protrusions (Fig. 6).

Extracellular fibrillar material appeared at the second week and increased with time. They might be short and arranged randomly, or collected into long bundles, paralleling to cytoplasmic microfilaments of the fibroblasts (Fig. 7). When the cultures were fixed and processed in the presence of ruthenium red, a large amount of proteoglycan existing as extracellular tiny particles of high electron density were showed by TEM. They also appeared as a mat-like layer covering the adherent stromal cells and surrounding hematopoietic islands (Fig. 8) under scanning electron microscope.

DISCUSSION

The formation of adherent layers in LTBMC reflects reconstruction of HIM in vitro. As to the composition of adherent layer, there have been many controversies, which were not only caused by difference in studying materials, but also came from disparity in nomenclature. We have not found any paper on LTBMC using fetal bone marrow cells in the literature. The developing HIM in fetal marrow is relatively immature albeit it can sustain active hemopoiesis during the late term. It is expected that fetal HIM contains the most important and fundamental components, and its very nature may be elucidated more clearly from LTBMC of fetal marrow by way of an ontogenetic approach.

In this study, fibroblast and MØ were found to be the main cellular components, together with reticular fibers, fibronection and proteoglycan, they constituted HIM in vitro and sustained hemopoiesis of at least myelomonocytic line. The existence of adipocyte and endothelial cell could not be confirmed by our cytochemical, immunocytochemical and ultrastructural observations. Some small round cells containing VIIIR:Ag were primitive megakaryoblasts certified by positive reactions with anti-GPIb, anti-GPIIb/IIIa antibodies.

Fibroblast characterized by large amounts of RER had been called epithelioid cell or reticular cell by some authors. By comparing the ratio of collagen type I to type III existing in bone marrow, LTBMC or simple fibroblast culture, Bentley [11] confirmed the fibroblast in LTBMC being identical to the reticular cell in bone marrow. So we prefer to call it fibroblast rather than other descriptive terms, and suppose its epithelioid appearance being caused by its particular microenvironment in culture.

The most intriguing cell type in LTBMC of fetal marrow was MØ, which varied in size markedly and usually contained many cytoplasmic vacuoles. Only by way of phagocytosis experiement and positive CMPase reactivity, it could be identified as MØ with certainty. Vacuolated MØs had beed reported by Chen [12], and Takahashi [13], in their ultrastructural studies of fetal bone marrow or skin of rat fetus respectively. So cytoplasmic vacuoles seem to be a characteristic feature of fetal MØs. We confirmed them being in the category of lysozyme by ultrastructural cytochemical demonstration of CMPase, a marker enzyme of lysozyme, and suspected that their presence in abundance were due to rapid metabolism and turnover of fetal tissues. MØ began to appear in fetal marrow before hemopoiesis occured there [12]. Is is expected that MØ played important roles in the establishment as well as maintenance of HIM. In hemopoietic islands of LTBMC MØs were found to associate with hemopoietic cells intimately, mimicking the central MØs of erythrocytic islands in bone marrow. However, no cell of erythrocytic line could be found in our study. Further study with some technical modification is indicated.

RESUMEN

Se establecieron cultivos de medula de feto humano por largos periodos de tiempo, desde 20 hasta 28 semanas de duración. Se realizaron estudios de la capa adherente por los siguientes métodos: 1) Tinción con Giemsa, Sudan IV e impregnación con plata Gomori. 2) Tinción inmunocitoquímica. 3) Experimentos de fagocitosis con ***Cándida albicans***. 4) Microscopia electrónica de barrido y de transmisión. 5) Reacciones ultraestructurales de Citidina monofosfatasa (CMPase) y mieloperoxidasa (MPO).

Los resultados indican que los fibroblastos y macrófagos (MØs) son los principales componentes de la capa adherente. La existencia de adipocitos y células endoteliales no pudo ser comprobada. Los fibroblastos se caracterizaron por presentar el reticulo endoplásmico rugoso muy desarrollado y gran formación de fibras reticulares y fibronectina (FN). Muchos MØs fueron capaces de engullir ***C. albicans***. La caracteristica morfológica

sobresaliente de los MØs fetales fue la presencia de gran número de vacuolas claras con actividad CMPase positiva. La matriz extracelular (ECM) presento una composición de FN, proteoglicano y fibras reticulares. Los fibroblastos y los MØs conjuntamente, componen el microambiente hematopoyético inductivo, en el cual, la proliferación y maduración de las células mielomonocíticas toman lugar.

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