The Roles of the Marginal Veins in the Differentiation of the Rat Hind Limb Bud: An Immunocytochemical Study

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Summary. Rat hind limb buds, aged between prenatal days 14 and 18, were used for electron microscopy and immunocytochemistry of fibronectin, laminin and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining indicative of DNA fragmentation.

Fibronectin and laminin were actively synthesized in the rough endoplasmic reticulum of epidermal cells in the apical ectodermal ridge between prenatal days 14 and 15, but most cells underwent apoptosis after prenatal day 15. As the regression of the apical ectodermal ridge progressed, mesenchymal cells associated with the marginal veins were successively incorporated into the endothelium devoid of the basal lamina. No mitotic figures of endothelial cells were recognized either in the marginal vein or in the surrounding growing capillaries. Extracellular matrix components connected the adjacent mesenchymal cells, with the endothelium of such vessels immunoreacting to fibronectin and laminin. In addition, fibronectin-immunoreactive networks among the interstices of the mesenchymal cell cords developed in the avascular zone between the epidermis and the growing capillaries at prenatal day 15, but became inconspicuous at prenatal day 16.

These results indicate that the apoptosis of the epidermal cells is the major reason for the regression of the apical ectodermal ridge, and that the capillary ingrowth from the marginal veins to the avascular zone is accelerated by transformation of mesenchymal cells to endothelial ones. Fibronectin and laminin seem to play crucial roles in capillary growth, especially in the adhesion between endothelial cells of the pre-existing vessels and mesenchymal cells.

The importance of epithelial-mesenchymal interactions in avian wing buds has been established by light microscopic immunocytochemistry (TOMASEK et al., 1982; CRITCHLOW and HINCHLIFFE, 1991, 1994). The apical ectodermal ridge (AER), a localized epidermal protuberance in the distal portion of avian wing buds, has been considered to play an inductive role in their proximodistal growth under the effects of epithelial-mesenchymal interactions (SAUNDERS, 1948; SAUNDERS and REUSS, 1974). CRITCHLOW and HINCHLIFFE (1994) demonstrated immunoreactions of certain extracellular matrix components such as fibronectin, laminin and type IV collagen along the epidermal-mesenchymal interface beneath the AER of the chick wing bud, and claimed that these components play crucial roles in the differentiation of the underlying mesenchyme. KELLEY and FALLON (1976) described that the AER undergoes regressive changes during the morphogenesis of avian wing buds and that this process is mainly due to necrosis of the epidermal cells. However, we cannot rule out the involvement of apoptotic cell death, as in the case of the apoptotic mesenchymal cell death during the formation of the interdigital spaces (MORI et al., 1995).

The marginal veins running along the AER in the underlying mesenchyme are presumed to be also involved in the proximodistal growth of avian wing buds. Furthermore, although it has been supposed that the existence of the marginal veins depends on the AER (FEINBERG and SAUNDERS, 1982), little is known regarding the roles of these vessels in mammalian limb buds. The roles of fibronectin and laminin as extracellular matrix components in the acceleration of capillary ingrowth have been pointed out in various developing organs (CLARK et al., 1982; WAKUI et al., 1990; HARA et al., 1994). However, no immunocytochemical analyses from such a viewpoint have been made on the neocapillarization of mammalian limb buds.

This paper mainly deals with the apoptotic cell death in the regression of the AER and the involvement of the marginal veins in the capillary ingrowth
of the rat limb bud.

MATERIALS AND METHODS

Animals

Hind limb buds of Wistar rats aged between prenatal days 14 and 18, which were equivalent to those of Swiss-Webster mice staged between 7 and 11 (WANEK et al., 1989), were isolated under general anesthesia by an inhalation of ethyl ether. Prenatal day 0 was defined as the morning on which sperm was first detected in a vaginal smear.

Electron microscopy

Specimens were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer for 4 h at 4°C, postfixed in 1% osmium tetroxide in the buffer, dehydrated in a series of acetone, and embedded in epoxy resin. Ultrathin sections were made on an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX electron microscope.

Scanning electron microscopy

Specimens were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer for 12 h at 4°C, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graded concentrations of butyl alcohol, freeze-dried with an EIKO 1D-2 freeze dryer, coated with platinum palladium, and examined in a Hitachi 5-4500 scanning electron microscope.

Light microscopic immunocytochemistry

Specimens were fixed in 3.7% paraformaldehyde in phosphate buffered saline (PBS) for 48 h at room temperature, dehydrated in graded concentrations of ethanol, and embedded in paraffin. Sections of approximately 4 μm in thickness were deparaffinized, rinsed in 0.1 M PBS, digested with 0.4% pepsin in a mixture of 0.01 N HCl and 0.1 M PBS for 2 h at 37°C, and treated with 0.3% H₂O₂ in absolute methyl alcohol for 20 min to reduce the endogenous peroxidase activity. After non-specific protein bindings were blocked with non-immune goat serum, sections were reacted to the primary fibronectin antibody at a dilution of 1:500 in 0.1 M PBS for 1 h at room temperature. Sections that were rinsed in 0.1 M PBS were reacted to FITC-conjugated goat-F(ab')2 fragment to rabbit IGG-P (ab')2 (Cappel, USA) at a dilution of 1:100 in 0.1 M PBS for 40 min at room temperature, mounted on slides, and examined in a Carl Zeiss LSM-410 confocal scanning laser microscope fitted with an Argon laser. In order to reduce random electronic noise, images were averaged after eight successive scans. The distance between each cutting optical plane was 0.25 μm, and three-dimensional images were constructed from twenty serial optical slices.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining indicative of DNA fragmentation

TUNEL staining was carried out according to the methods by GAVRIELI et al. (1992) and MORI et al. (1994) with slight modifications. Paraffin-embedded sections of approximately 4 μm in thickness were deparaffinized, rinsed in 0.1 M PBS, digested with 20 μg/ml proteinase K for 15 min at 37°C, and treated with 2.0% H₂O₂ for 5 min at 37°C. These sections were then immersed in a terminal deoxynucleotidyl transferase (TdT) buffer for 5 min at room temperature, and then incubated using an Apoptosis Detection Kit (Apop Tag, Oncor, Gaithersburg, MD, USA) for 60 min at 37°C. Finally, sections were reacted to antidigoxigenin-peroxidase for 30 min at room temperature and developed in a mixture of 0.05 % DAB and 0.01% H₂O₂. Control staining was performed by substituting distilled water for the TdT.

Immuno-electron microscopy

Specimens were fixed in a solution of periodate-lysine-paraformaldehyde (PLP) (McLEAN and NAKANE, 1974) in 0.1 M phosphate buffer for 6 h at 4°C. Approximately 40 μm-thick sections were made on a microslicer (DSK, Japan), and then reacted to the above-mentioned primary antibodies at a dilution of 1 : 1000 (fibronectin) and 1 : 200 (laminin) in 0.1 M PBS overnight at 4°C. After rinsing in 0.1 M PBS, sections were stained according to the BSA method. They were then postfixed in 1% osmium tetroxide in 0.1 M PBS for 15 min, dehydrated in graded concentrations of acetone, embedded in epoxy resin, cut into
Fig. 1. Apical ectodermal ridge (AER) of a rat hind limb bud on prenatal day 14. T trunk. ×200

Fig. 2. Some epidermal cells in the AER contain apoptotic bodies (arrows) on prenatal day 14. Mesenchymal cells (MC) aggregating below the AER are in contact with the basal lamina of the AER by their cytoplasmic projections (Insertion). One of the mesenchymal cells shows a mitotic figure (asterisk). ×4,000, Insert ×8,000
Fig. 3. Legend on the opposite page.
ultrathin sections, and examined in the electron microscope without counterstaining. For controls of the immunoreactions, the specificity was confirmed by substituting PBS or normal rabbit sera for the primary antisera.

RESULTS

Electron microscopy and immunocytochemistry of the AER

At prenatal day 14, the AER existed as a localized ridge-like thickening of the epidermis at the distal tip of the rat limb bud (Fig. 1). Most epidermal cells contained large granular structures including cellular debris or apoptotic body-like structures, and mesenchymal cells below the AER extended their cytoplasmic projections to the continuous basal lamina (Fig. 2). Mitotic figures of the mesenchymal cells were frequent (Fig. 2).

By light microscopic immunocytochemistry between prenatal days 14 and 15, the epidermal-mesenchymal interface and the vascular wall including that of the marginal veins were seen to immunoreact both to fibronectin and laminin (Fig. 3). Immunoreactions of both glycoproteins were preferentially localized in the rough endoplasmic reticulum of some epidermal cells inside the AER (Fig. 4a) and mesenchymal cells below the AER (Fig. 4b).

Regression of the AER

Cytoplasmic bodies, similar in morphological features to apoptotic ones, also immunoreacted to fibronectin and laminin (Fig. 4a). These bodies, heterophagocytosed by the adjacent epidermal cells in the AER, remarkably increased in number on prenatal day 15 (Fig. 5a). TUNEL staining revealed immunoreactive cells in the AER (Fig. 5b). Some mesenchymal cells around the marginal vein occasionally immunoreacted (Fig. 5b).

Neocapillarization from marginal vein

By light microscopic immunocytochemistry, the epidermal-mesenchymal interface and the vascular wall including that of the marginal veins were seen to immunoreact both to fibronectin and laminin between prenatal days 14 and 16 (Figs. 3, 6, 9, 10). Growing capillaries enclosing a slit-like lumen extended from the pre-existing vessels—including the marginal veins—to the avascular zone after prenatal day 15 (Fig. 6).

Between prenatal days 14 and 15, the marginal veins, which run in the mesenchyme almost parallel to the AER and consist of a thin endothelium enclosing a wide lumina, were closely associated with an abundance of mesenchymal cells (Fig. 7). Several morphological changes in the marginal veins became pronounced as the salient regressive changes appeared in the AER from prenatal day 15: These included the appearance of cytoplasmic vacuoles in the endothelial cells (Fig. 7a) and the incorporation of neighboring mesenchymal cells, which were occasionally in contact with each other, into the endothelium devoid of the basal lamina (Fig. 7b). Mitotic figures of the mesenchymal cells were occasionally found, while no mitotic endothelial cell figures were observed in our samples (Fig. 7b).

By immuno-electron microscopy, immunoreactions of both glycoproteins were seen to be preferentially localized along the basal side of the endothelium of the marginal veins, on the endothelial cell surface, in the rough endoplasmic reticulum of the mesenchymal cells, and on the extracellular matrix components connecting the adjacent mesenchymal cells (Fig. 8).

Fibronectin-immunoreactive strands of the avascular zone

Fibronectin-immunoreactive strands extending from the epidermal-mesenchymal interface to the growing capillaries among the mesenchymal cell cords through the avascular zone appeared on prenatal day 15 (Figs. 3a, 6a, 10, 11) and had almost disappeared by prenatal day 16 (Fig. 9a). Such profiles of the immunoreactivity were not clearly immunoreactive for laminin (Fig. 6b).

Fig. 3. Longitudinal sections of a rat hind limb bud on prenatal day 14. a. Immunoreactions of fibronectin are seen along the epidermal-mesenchymal interface (EMI) and vascular wall including that of the marginal veins (MV). Fibronectin-immunoreactive strands extend from the epidermal-mesenchymal interface (EMI) to the vascular wall through the avascular zone (arrows). b. Immunoreactions of laminin in an adjacent section are seen along the epidermal-mesenchymal interface and the vascular wall, but the immunoreactive strands shown in Figure 3a are not apparent. c. Control section treated only with PBS. a–c: ×300
Fig. 4 a. Immunoreactions of fibronectin in the nuclear envelope of epidermal cells (ED) and along the basal lamina (BL) of the AER on prenatal day 15. Presumed apoptotic bodies (arrows) in the epidermal cells are also immunoreacted. Without counterstaining. ×10,000. b. Cisterns of the rough endoplasmic reticulum (arrows) in the mesenchymal cells (MC) below the AER and certain extracellular matrix components (arrowheads) show immunoreactions of fibronectin. Without counterstaining. ×7,000
Fig. 5  

**a.** Apoptotic bodies remarkably increase in number in epidermal cells of the AER on prenatal day 15. *BL* basal lamina. ×8,000.  

**b.** Some epidermal cells (*arrows*) in the AER are positively immunoreacted by the TUNEL method on prenatal day 15. A mesenchymal cell (*MC*) adjacent to a marginal vein (*MV*) is also TUNEL-positive. *EMI* epidermal-mesenchymal interface. ×1,000
Fig. 6. Legend on the opposite page.
Differentiation of Rat Limb Bud

Fig. 6. Transverse sections of a rat hind limb bud on prenatal day 15. a. Immunoreactions of fibronectin are seen along the epidermal-mesenchymal interface (EMI) and vascular wall of marginal veins (MV) and growing capillaries developing below the avascular zone. Each digital precartilage is also immunoreacted (asterisks). Fibronectin-immunoreactive strands through the avascular zone as shown in Figure 3a have become much more pronounced. b. Immunoreactions of laminin in an adjacent section are seen along the epidermal-mesenchymal interface (EMI) and vascular wall. Growing capillaries enclosing a slit lumina arise from pre-existing vessels (arrows). No digital precartilages is immunoreacted (asterisks). c. Control section treated only with PBS. ×100

Fig. 7. Marginal veins of rat hind limb buds on prenatal day 15. a. An endothelial cell (EC) of the marginal vein (MV) includes cytoplasmic vacuoles. The endothelium is closely associated with mesenchymal cells (MC) forming solid cell cords. MV marginal vein. ×3,000. b. A mesenchymal cell (MC1) extends a cytoplasmic projection to discontinuous portions of the endothelium (arrow). Another cell (MC2) shows a mitotic figure. ×2,500
DISCUSSION

The roles of various extracellular matrix components including fibronectin and laminin and have been demonstrated in the morphogenesis of avian wing buds (LINDER et al., 1975; DESSAU et al., 1978, 1980; NEWMAN et al., 1981; KOSHER et al., 1982; SOLURSH et al., 1982; TONASEK et al., 1982; CRITCHLOW and HINCHLIFFE, 1991, 1994). Our immuno-electron microscopy of the rat limb bud indicates that epidermal cells in the AER and mesenchymal cells below the AER are actively involved in the production of these glycoproteins. We found no inconsistencies with the consideration that these glycoproteins are involved in the formation of the digital precartilage under the effects of reciprocal epithelia-mesenchymal interactions in the avian wing bud as already discussed by NEWMAN et al. (1981) and TONASEK et al. (1982).

Based on the ultrastructure and immunocytochemical labelings of the DNA fragmentation, we confirmed that epidermal cell death by apoptosis in the AER is the major reason for the regression of the AER after prenatal day 15. FEINBERG and SAUNDERS (1982) described how the maintenance of the marginal veins depends on that of the AER in the avian wing bud. In our samples, the regressive changes of the marginal veins became pronounced in proportion to those of the AER after prenatal day 15. During this period, endothelial cells of the marginal veins showed degenerative changes resulting in the discontinuity of the endothelium (see Fig. 7a). An abundance of mesenchymal cells, which were in contact with each other and occasionally formed solid cell cords, existed around the marginal veins. The cytoplasmic projections of these mesenchymal cells were occasionally incorporated into the discontinuous endothelium of the marginal veins (see Fig. 7b).

The term "vasculogenesis" is reserved for de novo vessels in very early embryos such as the dorsal aortae and the posterior cardinal vein (WAGNER, 1980), and is generally defined as the integration of mesenchymal cells as vasoformative ones into pre-existing vessels, whereas the term "angiogenesis" is generally used for the process by which endothelial sprouts arise from pre-existing vessels (AUSPRUNK and FOLKMAN, 1977). The transformation of mesenchymal cells to vasoformative ones has been described in embryonic tissues (FUJIMOTO et al., 1987; HARA et al., 1994), in wound healing tissues (MORI et al., 1992; HIRATA et al., 1994) and in tumors (HAMMERSSEN et al., 1985). The capillary ingrowth arising from the marginal

Fig. 8. Mesenchymal cells (MC) associated with a marginal vein (MV) are in contact with each other or with the endothelium by fibronectin-immunoreactive extracellular matrix components (arrowheads) on prenatal day 15. The nuclear envelope of the mesenchymal cells (MC) shows immunoreactions for fibronectin (arrows). Without counterstaining. \( \times 8,000 \)
Fig. 9. Transverse sections of a rat hind limb bud on prenatal day 16. a. Immunoreactivity for fibronectin is seen along the epidermal-mesenchymal interface (EMI) and the vascular wall of marginal veins (MV) and growing capillaries. Each digital precartilage is also immunoreacted (asterisks). Fibronectin-immunoreactive strands as shown in Figures 3a and 6a have become inconspicuous. b. Immunoreactivity for laminin in an adjacent section is localized along the epidermal-mesenchymal interface (EMI) and the vascular wall, but is not pronounced on each digital precartilage (asterisks). c. Control section treated only with PBS. a–c: ×80
veins in a manner suggestive of vasculogenesis is considered to be an initial event in the neocapillarization of the avascular zone in our samples since we did not encounter any mitotic figures of endothelial cells of the pre-existing vessels, and we agree with the reports by Seifert et al. (1992) and Brand-Saberi et al. (1995) that the neocapillarization by transformation of mesenchymal cells to endothelial ones is more prevalent in the avascular zone, while that in the manner of sprouts from the pre-existing vessel frequently occurs beneath the avascular zone in the chick wing bud.

The extracellular matrix components of contact areas between the adjacent mesenchymal cells and between the mesenchymal cell and endothelial cell showed intense immunoreactions of fibronectin and laminin. Bissel et al. (1987), Grant et al. (1989) and Martinez-Hernandez et al. (1991) reported the involvement of these glycoproteins in capillary ingrowth in *in-vivo* and *in-vitro* specimens. Loeber and
Runyan (1990) and Wakui et al. (1990) have noted that fibronectin increases capillary ingrowth during wound healing. Taking these reports into consideration, it is likely that these glycoproteins play the crucial roles in the capillary ingrowth from the marginal veins.

One of the unique features obtained from the present immunocytochemical study was the appearance between prenatal days 14 and 15 of fibronectin-immunoreactive strands extending from the epidermal-mesenchymal interface to the capillary wall through the avascular zone (see Figs. 3, 6a, 10, 11). These strands are considered to manifest fibronectin-rich extracellular matrix networks among the interstices between the mesenchymal cell cords beneath the epidermis. The existence of similar strands called "dorso-ventral fibrils", which extend to the dorso-ventral direction throughout the mesenchyme of the chick wing bud and immunoreact to type I collagen, tenascin and fibronectin, has been already reported by Hurle et al. (1989). They supposed that these components might play a role in the formation of the digital plate. It is adjacent, at present, to presume the possible roles of our fibronectin-immunoreactive extracellular matrix networks localized in the avascular zone. It seems likely that they are continuously constructed by the mesenchymal cells which aggregate in rows toward the epidermis through the avascular zone, and are also involved in the regulation of proliferations and migrations of the mesenchymal cells. If this is true, such a regulatory process may be important for the normal growth of the rat hind limb bud. This assumption arguably supports the concept that several kinds of glycoproteins in extracellular matrix components might play a role in the formation of the digital plate. It is difficult, at present, to presume the possible roles of our fibronectin-immunoreactive extracellular matrix networks localized in the avascular zone. It seems likely that they are continuously constructed by the mesenchymal cells which aggregate in rows toward the epidermis through the avascular zone, and are also involved in the regulation of proliferations and migrations of the mesenchymal cells. If this is true, such a regulatory process may be important for the normal growth of the rat hind limb bud. This assumption arguably supports the concept that several kinds of glycoproteins in extracellular matrix components provide a scaffold during organogenesis (Dessau et al., 1978; Hurle et al., 1989; Baloch et al., 1992).

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REFERENCES


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