Effect of Fetal Serum on Fibroblast Pericellular Matrix Formation

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Hyaluronic acid (HA)-dependent pericellular matrices (PCM) play a role in embryonic differentiation of mesodermal cells. Fetal fibroblasts have significantly larger PCMs than postnatal fibroblasts. To determine if this property is intrinsic to fetal fibroblasts or induced by factors in the fetal environment, we studied the effect of fetal bovine serum (FBS) of varying gestational age on human fetal, newborn, and adult fibroblast PCM formation. Cultured human fetal, newborn, and adult fibroblasts were plated in triplicate at a density of 1×10^5 cells and incubated in medium alone, medium containing 10% pooled FBS, or FBS from the first, second, or third trimesters. The cells were photographed and morphometric analysis of PCM was performed by the erythrocyte exclusion technique. PCM size was expressed as a ratio of the maximal width of the cell matrix to the maximal width of the cell. The unpaired Student's t test was used for statistical analysis. The earlier the gestational age of FBS used, the larger the PCM observed in fetal and newborn fibroblasts. The PCM of fetal fibroblasts was significantly larger ($P < 0.001$) than that of newborn and adult fibroblasts at each gestational age of FBS tested (fetal $\geqslant$ newborn $>$ adult). Medium containing pooled FBS caused a significant ($P < 0.001$) increase in PCM size in all cell lines compared with serum-free medium. There are both intrinsic and extrinsic factors which affect PCM size. These factors which affect HA-dependent PCM size may contribute to a permissive microenvironment for cell migration, proliferation, and development which may be important for scarless fetal wound repair. © 1996 Academic Press, Inc.

uronic acid (HA) [1–5]. The result is a restoration of skin integrity by regeneration of normal dermal structures without scar formation [6]. The mechanisms that underlie this unique fetal response to injury are unknown.

HA is thought to play a crucial role in the extracellular matrix of healing fetal wounds [7, 8]. The exact source and function of HA in fetal wounds remains undefined, but it is known that elevated levels of HA are present in fetal wounds up to 21 days postwounding [5]. During development the presence of extracellular matrix rich in HA has been repeatedly correlated with cell proliferation and migration, whereas HA-deficient matrix has been associated with cessation of these activities and expression of a more differentiated phenotype and fibroplasia [9–11].

We have previously demonstrated that human fetal and postnatal fibroblasts elaborate an HA-dependent pericellular matrix (PCM) [12]. Studies have shown that PCMs *in vitro* mimic the synthesis and assembly of PCMs *in vivo* [13–15]. PCMs are thought to play a role in differentiation, cellular motility, and proliferation and may modulate cellular–extracellular matrix interactions. Elaboration of a larger size PCM and a greater percentage of fibroblasts expressing PCMs appear to be properties intrinsic to fetal fibroblasts [12]. However, we postulate that PCM expression might also be influenced by extrinsic factors present in the fetal environment. In order to evaluate the influence of the fetal environment on expression and size of the PCM, we studied the effect of exposure to fetal bovine serum (FBS) of varying gestational age on PCM size in human fetal, newborn, and adult fibroblasts.

INTRODUCTION

Fetal wound healing is characterized by an accelerated rate of healing, an absence of inflammatory infiltrate, and the rapid and organized deposition of extracellular matrix with high and persistent levels of hyal-

METHODS

Cell lines and culture conditions. Normal human dermal fibroblasts obtained from a 12-week-gestation fetus (WAS 1), newborn foreskin (CCD-43SK), and 44- and 66-year-old adults (CCD-866SK, CCD-944SK) were obtained from American Type Culture Collection (Rockville, MD) and established in culture in Eagle's minimum essential medium (MEM) with nonessential amino acids, Earle's basic salt solution and 10% FBS (Hyclone Laboratories, Inc., Logan, UT). Fibroblast cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Fibroblasts were used between the 3rd and the 18th passage.
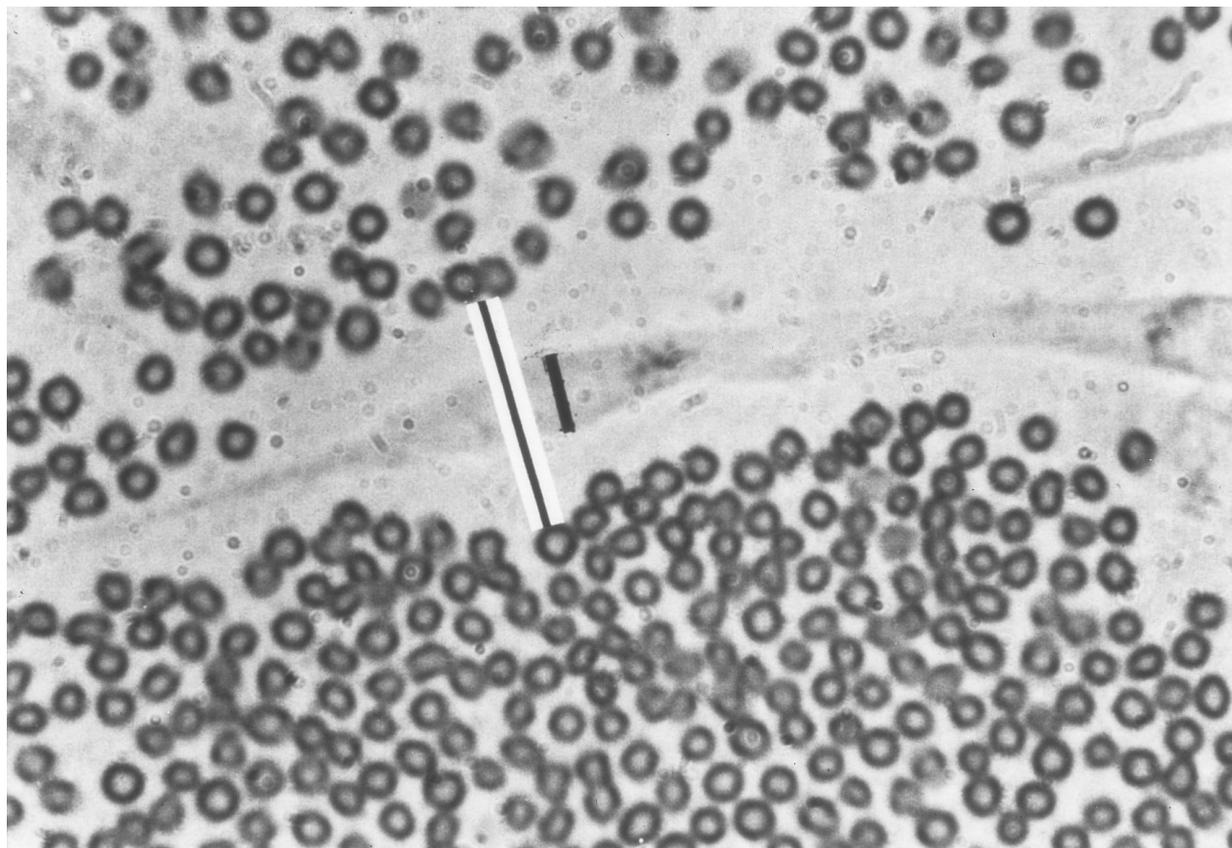
FIG. 1. The pericellular matrix to cell body width ratio (PCM/CB ratio) is derived from the measurement of the maximal PCM width (white line) and the maximal fibroblast CB width (black line). Fixed horse erythrocytes allows measurement of the widths via the cell exclusion technique. The horse red blood cells are added to the medium after 24 hr of incubation and allowed to settle for 15 min. Fibroblasts are photographed using an inverted phase-contrast microscope at $100\times$ magnification.

Fibroblast subcultures were plated in triplicate in 35-mm plates at low density (1×10^5 cells per well). Fibroblasts were released from culture with calcium- and magnesium-free phosphate buffered saline (PBS) and 0.1% trypsin EDTA, resuspended, washed, and resuspended in MEM. Viable cells were counted on a hemocytometer using trypan blue exclusion. The fetal, newborn, and adult fibroblast cell lines were established in each well with MEM alone, 10% pooled FBS, or 10% FBS from the first, second, or third trimesters (Hyclone Laboratories, Inc.). The medium was supplemented with nonessential amino acids and 1% penicillin-streptomycin in 35-mm 12-well plates for 24 hr prior to PCM assay.

Particle exclusion assay for pericellular matrix. Cells with large amounts of surface-associated HA *in vitro* often exhibit prominent pericellular coats which can be visualized by the cells' ability to exclude particles such as erythrocytes. The outline of the coat is revealed as a halo around individual cells in a particle exclusion assay (Fig. 1). Horse erythrocytes (GIBCO, Grand Island, NY) were fixed in 1.5% formalin, washed, and stored in calcium- and magnesium-free PBS at 4°C until use.

The fibroblasts were incubated for 24 hr at 37°C with 5% CO_2 in medium containing MEM alone, MEM containing 10% pooled FBS, or MEM containing 10% FBS from the first, second, and third trimesters. Fixed horse erythrocytes (1×10^8) were then added to each well as previously described [16]. The particles were allowed to settle on the microscope stage for 15 min. The cells were photographed using a Nikon Diaphot inverted phase-contrast microscope.

Morphometric analysis of pericellular matrices. Photomicrographs (about 20 per cell line) of randomly selected cells (approximately 50–80 fibroblasts per cell line) were printed at the same magnification ($100\times$). In order to be included for morphometric analysis the entire fibroblast had to be seen without crossing fibroblasts or areas of continuity with adjacent PCMs. The erythrocytes are excluded by the pericellular matrix, allowing measurement of the maximal width of the pericellular matrix as previously described [12]. Pericellular matrix size was expressed as a ratio of the maximal width of the PCM to the maximal width of the cell body (CB). A ratio of >1.5 indicates a reliably detectable PCM/CB ratio (Fig. 1).

Data were summarized as the mean $\pm$ the standard deviation. The unpaired Student's t test was used for statistical analysis. Differences were considered significant at $P < 0.05$.

RESULTS

Fibroblasts from each cell line (fetal, newborn, and adult) demonstrated detectable pericellular matrices *in vitro* (Fig. 1). There was no significant difference in cell body width among fetal (2.46 ± 0.09 mm, mean $\pm$ SEM), newborn (2.65 ± 0.12, $P = 0.02$), and adult (2.67 ± 0.1, $P = 0.1$) fibroblasts. Exposure of fibroblasts to medium with pooled FBS resulted in significant increases in the PCM/CB ratio in each cell line compared to that of serum-free medium ($P < 0.001$) (Fig. 2). PCM/CB ratios increased in the presence of pooled FBS in an age-dependent manner. Fetal fibroblasts demonstrated larger increases in PCM/CB ratios than newborn fibroblasts, which had larger PCM/CB ratios than adult fibroblasts.

Exposure of fibroblasts to medium containing FBS from each trimester resulted in larger PCM/CB ratios in fetal and newborn cell lines (Fig. 3). Exposure of

fetal and newborn fibroblasts to medium containing first trimester FBS resulted in larger PCM/CB ratios than that in medium containing FBS from the second or third trimesters ($P < 0.001$). No significant changes in the adult fibroblast PCM/CB ratio were noted with FBS of any trimester. Fetal fibroblasts had the largest PCM/CB ratio compared with either newborn or adult fibroblasts ($P < 0.001$) at each gestational age of FBS tested. The larger PCM/CB ratio of fetal fibroblasts became more pronounced with exposure to medium containing first trimester FBS.

DISCUSSION

Factors in the fetal environment appear to be capable of influencing the size of fibroblast PCMs, as the earlier the gestational age of FBS to which fibroblasts are exposed, the larger the PCM observed. Previous work established intrinsic differences in PCM size and expression between fetal and postnatal (newborn and adult) fibroblasts [12]. Intrinsic to the fetal fibroblast is the production of significantly larger PCMs than those of postnatal fibroblasts. This pericellular matrix is HA-dependent and reliant upon HA-binding protein for assembly [12]. The results of the present study suggest that in addition to the intrinsic ability of fetal fibroblasts to elaborate HA-dependent PCMs, extrinsic factors in the fetal environment influence HA-dependent PCM size.

There are several possible causes for the influence of earlier gestational age FBS on the PCM/CB ratio including increased production, decreased breakdown, or factors present which stabilize the PCM. The effects of FBS on the HA-dependent PCM may be related to another factor, HA-stimulating activity (HASA) [17].
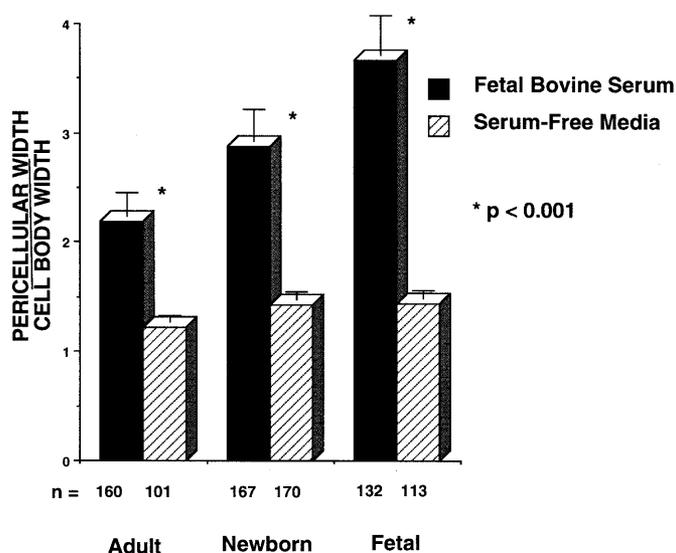
FIG. 2. Exposure of adult, newborn, and fetal fibroblasts to pooled samples of FBS resulted in significant increases in the PCM/CB ratio in each cell line ($P < 0.001$). This effect was age-dependent, as the fetal fibroblasts demonstrated the largest increases in the PCM/CB ratio and the adult fibroblasts the smallest.
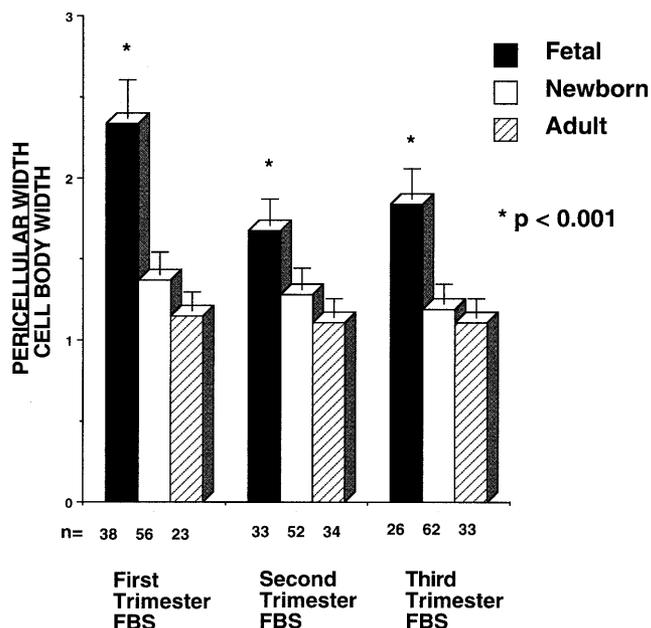
FIG. 3. The effect of gestational age of the FBS on the PCM was evaluated by comparison of the PCM/CB ratio of fetal, newborn, and adult fibroblast cell lines after incubation with FBS of varying gestational age: first trimester (1–3 months), second trimester (5 months), and third trimester (8 months). The PCM/CB ratio of fetal fibroblasts was significantly larger ($P < 0.001$) than newborn and adult fibroblasts at each gestational age of FBS tested. The larger PCM/CB ratio of fetal fibroblasts became more pronounced with earlier gestation FBS.

Longaker *et al.* postulated that the HA-rich ECM seen in fetal wounds was the result of HA-stimulating activity of fetal sera, wound fluid, and amniotic fluid [17, 18]. However, peak HASA occurred in mid-gestation in fetal bovine sera, unlike the peak effects on the PCM/CB ratio observed with first trimester FBS [18]. Schor has found that fetal fibroblasts produce a substance, migration stimulating factor, that stimulates synthesis of HA [19]. This protein is found in medium conditioned by fetal fibroblasts (FFCM) and may act in an autocrine fashion. FFCM increases the percentage of cells that express detectable PCMs in fetal, newborn, and adult cell lines [12]. In addition, decreased degradation of HA may result from either lower hyaluronidase activity or a substance that accelerates hyaluronidase breakdown. Lower hyaluronidase activity in fetal wounds has been reported by Mast *et al.* [8]. Another factor present in serum, inter-α-inhibitor, has been reported to stabilize HA-dependent PCM in normal human fibroblasts [20]. This substance has been isolated from adult human serum and if present in high concentration in earlier gestation, fetal sera could account for the larger PCMs we observed in our experiments. In support of this, Blom *et al.* has demonstrated that pooled fetal bovine serum will stabilize fibroblast PCMs at concentrations 100 times lower than the concentration of adult bovine serum [20]. FBS may contain more inter-α-inhibitor or other factors than postnatal sera which are responsible for the larger PCM observed with exposure to fetal serum. The ability of the FBS to in-

duce larger PCMs seems to diminish postnatally. Incubation with first trimester FBS causes a significant increase in the PCM/CB ratio of fetal and newborn fibroblasts. This response to FBS is absent in adult fibroblasts. The size of the HA molecule produced by fetal fibroblasts has also been shown to be larger than the HA molecules produced by postnatal fibroblasts which may influence PCM size [21].

Exposure to pooled FBS resulted in larger PCM/CB ratios in all three cell lines compared with the PCM/CB ratios obtained after exposure to first, second, or third trimester FBS. Conditions may have varied because different lots of fetal sera were used for pooled and individual trimester FBS. Despite the differences in PCM/CB ratio observed with pooled versus the separate trimester FBS, the data support the conclusion that extrinsic factors present in fetal serum may influence PCM size and expression.

The HA-dependent PCMs observed in fibroblasts may be an important factor in cell–extracellular matrix interactions. The larger size and greater percentage of cells expressing PCMs in fetal fibroblasts may reflect one aspect of a unique fetal fibroblast phenotype [12]. The consistent observation of prolonged and elevated levels of HA in fetal wound ECM may be a clue to a mechanism of scarless fetal wound repair. In order for HA to influence interactions between fibroblasts and the ECM, organization of HA into PCMs may be essential in growth factor-binding and presentation, collagen assembly, and cell–cell interactions. A more fundamental understanding of the factors responsible for the fetal fibroblast phenotype may allow the development of strategies to influence postnatal fibroblast behavior. Conversion to a fetal fibroblast phenotype in postnatal fibroblasts may allow a more regenerative response to wounding and minimize scar formation.

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