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The Extracellular Matrix of the Fetal Wound: Hyaluronic Acid Controls Lymphocyte Adhesion

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Adhesive interactions between lymphocytes and components of the extracellular matrix (ECM) within a wound environment play a crucial role in determining the inflammatory response following tissue injury. In fetal wounds the extracellular matrix is composed predominantly of hyaluronic acid. Within this environment the inflammatory reaction as a result of injury is minimal. We propose that this lack of an inflammatory cell response in the fetal wound is due to the high levels of hyaluronic acid within the ECM and the inability of lymphocytes to adhere to this matrix component. Therefore, we examined the adhesive properties of fetal lymphocytes to fibronectin, vitronectin, collagen types I, III, IV, V, and hyaluronic acid—ECM components involved in fetal and adult wound environments. Fetal lymphocytes from both spleen and thymus demonstrated significant binding capabilities to fibronectin, vitronectin, and collagen types I and III. No intrinsic binding capabilities were detected to hyaluronic acid. Adhesion was not affected by the addition of IL-1, IFN-γ, or phorbol dibutyrate. The inability of lymphocytes to adhere to hyaluronic acid helps to explain the lack of inflammation found in fetal wounds and serves to demonstrate the importance of ECM–lymphocyte interactions in determining the inflammatory response during fetal wound healing.

INTRODUCTION

Extensive wound healing research has clearly documented the remarkable characteristics of fetal tissue repair following injury [1–10]. Perhaps the most striking aspect of these investigations has been the finding that fetal wounds heal without an inflammatory response and generate a "scarless" repair marked by tissue regeneration and organized structural integrity rather than the disorganized scar found in adult wound healing. Since inflammation is such a crucial component of adult wound healing, its absence in the fetal response to injury may be an important factor in this process of scarless tissue repair [11–13].

The importance of lymphocytes, and in particular T-lymphocytes, in the process of adult wound healing and their absence in fetal wounds has recently been reported [14–16]. T-lymphocytes appear to be crucial to collagen formation and an adult-like scar response. In order to mediate an inflammatory response as the first stage of tissue repair lymphocytes must be able to interact with the glycoproteins and glycosaminoglycans that compose the extracellular matrix (ECM) of the wound environment. Though the fetal wound environment contains the same ECM glycoproteins as the adult wound (fibronectin, vitronectin, and collagen subtypes), the major component of the fetal wound is the glycosaminoglycan hyaluronic acid [1, 2, 5–7, 17, 18].

Hyaluronic acid is distributed in the extracellular matrix of most tissues and is particularly concentrated in developing or regenerating tissues [19]. Though present transiently in adult wounds, it dominates the fetal wound matrix [1, 2, 5, 9]. It is a simple glycosaminoglycan composed of repeating units of *N*-acetylglucosamine and glucuronic acid that can form a randomly coiled macromolecule with up to 25,000 disaccharide pairs and no protein backbone. This structure can occupy a large solutional domain and is thought to create hydrated pathways throughout the stabilized matrix that facilitate mesenchymal or stromal cell migration during embryogenesis and tissue regeneration [20].

Given the importance of lymphocyte interactions with ECM we proposed that the lack of inflammation within a fetal wound was due to an inability of fetal lymphocytes to bind to the fetal wound matrix and, in particular, hyaluronic acid. Therefore, the present studies were undertaken to examine the adherence properties of fetal lymphocytes to the individual components of the ECM and to assess the effects of inflammatory cytokines in modulating these adhesive interactions.

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METHODS

Sixteen-day gestational aged fetuses (term = 21 days) were isolated from time-dated pregnant CD1 mice (n = 80) (Charles River Laboratories, MD) and placed in RPMI containing 10% fetal calf serum (FCS) at 4°C. Spleen and thymus tissues isolated from each fetus and pooled in groups of eight pregnancies were homogenized and resuspended in RPMI–10% FCS at 4°C. Contaminating erythrocytes were lysed with Tris-buffered ammonium chloride for 15 min followed by two washes in RPMI–10% FCS at 4°C. Suspended lymphocytes were then isolated from debris by filtration through sterile gauze, washed twice in RPMI–10% FCS, and resuspended at a final dilution of 5×10^7 cells/ml.

Ninety-six-well microculture plates (Corning, Corning, NY) were precoated and air-dried overnite with fibronectin (5 μg), vitronectin (0.5 μg), laminin (5 μg), collagen type III (5 μg), collagen type V (5 μg) (Telios Corp., San Diego, CA), collagen type I (5 μg), collagen type IV (5 μg), and hyaluronic acid (5 μg) (Sigma Corp, St. Louis, MO) at 6 wells/individual component. Wells were then blocked with 1% bovine serum albumin prior to the adhesion assay.

The adhesion assay was performed by adding 5×10^6 cells/well and incubating the plates for 4 hr at 37°C in 5% CO_2. The wells were then filled with RPMI–10% FCS, and the nonadherent cells were removed by inverted centrifugation at 50g for 5 min. Adherent cells were fixed with 1% glutaraldehyde (Sigma) for 15 min. The wells were then washed with Hanks' balanced salt solution twice and air dried.

Quantification of adherent cell binding was determined using a modification of the crystal violet staining technique [21, 22]. Cells were stained by the addition of 100 μl of a 0.1% crystal violet solution buffered to pH 6.7 for 24 hr. Plates were then washed in distilled water for 35 min and air-dried. Dye was solubilized with 35 μl of 10% acetic acid solution for 30 min followed by the addition of 65 μl of 95% ethanol for 2 hr. The optical density of released dye was measured using a Dynatech MR 600 microplate reader (Dynatech Laboratories, Inc., Alexandria, VA) with the Biolynx Program on a HP-Vectra QS/20 computer (Hewlett–Packard Corp., Sunnyvale, CA). The test filter was set at 590 nm and the reference filter at 700 nm.

For each experiment a standard binding curve was established by manually counting six control wells precoated with Peptite 2000 (Telios Corp) containing serial dilutions of lymphocytes prior to destaining and determination of absorbance. A standard curve was then constructed from the cell counts and the corresponding absorbances of the calibration wells. Adherent cell binding in the experimental plates was then determined using the regression values acquired from the standard curve (Fig. 1). All assays were done in duplicate.

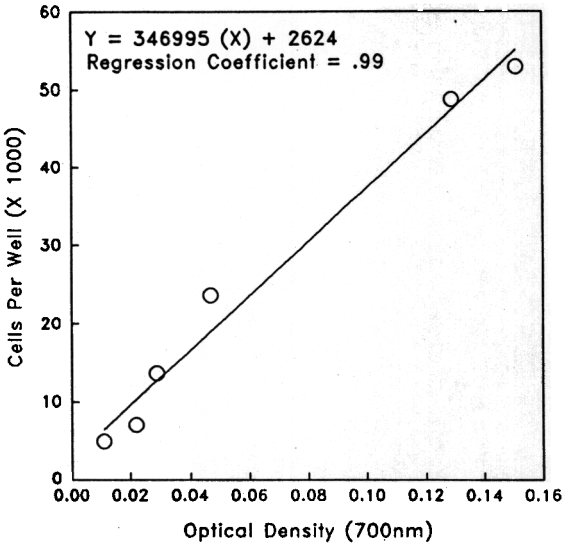

FIG. 1. Standard binding curve of fetal lymphocytes determined with crystal violet staining.

To determine the possible effects of inflammatory cytokines on binding, lymphocytes were cocultured with IL-1 (1 unit/ml), IFN-γ (100 units/ml) (R&D Systems, Minneapolis, MN), and phorbol dibutyrate (PDB, 50 ng/ml) (Sigma) during the 4-hr adhesion period. A total of three experiments for each cytokine with six wells per matrix component were performed.

Percentage adhesion (±SEM) was calculated with data analysis by one-way ANOVA and P values by unpaired Student's t test.

RESULTS

Adhesion to various components of the extracellular matrix by thymic lymphocytes harvested from Day 16 gestational aged murine fetuses is shown in Fig. 2. Maximum fetal thymocyte binding was demonstrated to fibronectin at 6.6 ± 1.0% of the cell population. Thymocyte binding to vitronectin was 3.8 ± 0.6%. Differential binding to the various collagen subtypes was exhibited with 5.8 ± 1.6% of the thymocyte population binding to collagen type I and 3.1 ± 0.6% binding to collagen type III. No significant binding could be determined to collagen types IV and V. Minimal binding was detected to hyaluronic acid by fetal T-cells (1.6 ± 0.6%) ($P < 0.001$).

Since the fetal spleen is also a source of developing lymphocytes, Day 16 splenocytes were harvested and tested for their adhesive capabilities (Fig. 3). Markedly enhanced binding to fibronectin involving 34.2 ± 5.2% of the cell population was detected, while binding to vitronectin was 14.1 ± 1.9%. Adherence to collagen type I was shown by 25.3 ± 3.2% of the splenocytes and to collagen type III by 17.5 ± 2.9% of the cells. A small population of cells (7.8 ± 1.4%) adhered to collagen type

IV. Once again, minimal binding was detected to hyaluronic acid by lymphocytes developing in the spleen (2.5 ± 1.7%) ($P < 0.001$).

The addition of IL-1, IFN-γ, or PDB for 4 hr did not effect the binding of fetal thymic or splenic lymphocytes to any of the extracellular matrix components (data not shown).

DISCUSSION

The data from these experiments demonstrate that fetal lymphocytes express the necessary receptors for and are capable of binding to a number of glycoproteins within the extracellular environment of the fetal wound and that such adhesive capabilities are present early in the ontogeny of these cells. However, there is no intrinsic binding capability of these lymphocytes to the dominant component of the wound matrix—hyaluronic acid. Since the presence of hyaluronic acid is the major biochemical difference between fetal and adult wound environments, these findings support the hypothesis that the presence of HA within the fetal wound plays a role in lymphocyte adhesion. Without the ability to adhere to the interstitial matrix fetal lymphocytes are unable to migrate into the zone of tissue injury.

The exact role for hyaluronic acid and the reason for its prolonged existence within the fetal wound environment remains unclear, although it is certain to be multifaceted. It has been shown to have a direct effect on modulating the overall wound healing response. If the hyaluronic acid content in the fetal wound is decreased, an adult-like response with leukocyte infiltration, disorganized collagen deposition, and neovascularization develops [23]. On the other hand, if hyaluronic acid is added to an adult wound, fibrosis and scar formation can be decreased [24, 25]. There appears to be a direct association between the extracellular matrix levels of this glycosaminoglycan and the degree of lymphocyte infiltration and inflammation within a wound environment.

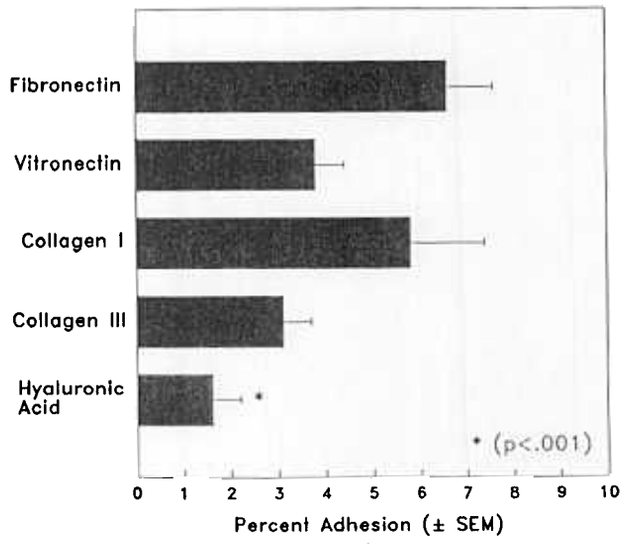

FIG. 2. Fetal thymic lymphocyte binding to components of the extracellular matrix (% total cell population).

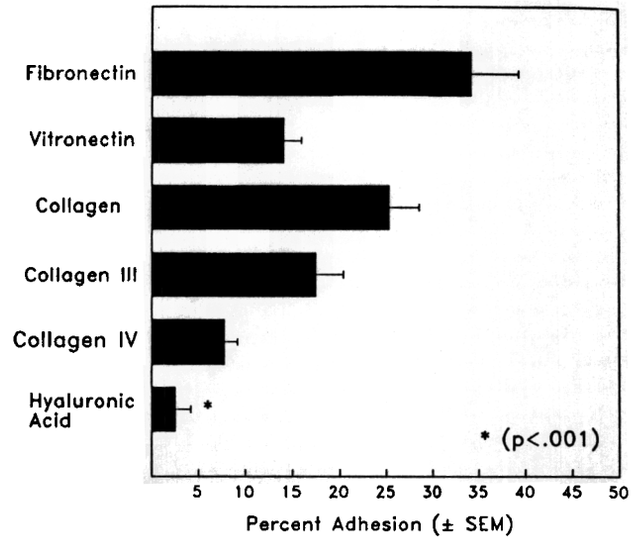

FIG. 3. Fetal splenic lymphocyte binding to components of the extracellular matrix (% total cell population).

The mechanism by which the level of hyaluronic acid within a wound environment affects the inflammatory reaction remains unknown. Since fetal lymphocytes fail to bind to immobilized hyaluronic acid even when stimulated with inflammatory cytokines, they cannot be recruited into or migrate through a zone of tissue injury in the fetus. Such processes require adhesive interactions with elements of the ECM, of which hyaluronic acid is the dominant component. Thus, hyaluronic acid may prevent an inflammatory response by providing an inadequate matrix for lymphocyte adhesion and migration.

We conclude that the lack of binding of fetal lymphocytes to hyaluronic acid demonstrates the importance of lymphocyte–ECM adhesive interactions in the wound healing response. The minimal inflammatory response characteristic of the fetal wound environment may be due in part to the composition of the extracellular matrix, as the presence of hyaluronic acid combined with the inability of fetal lymphocytes to adhere to this matrix component ultimately leads to tissue regeneration rather than inflammation and scar formation.

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