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Chemoattractant activity of degradation products of fetal and adult skin extracellular matrix for keratinocyte progenitor cells

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Abstract

Biologic scaffolds composed of naturally occurring extracellular matrix (ECM) have been utilized as templates for the constructive remodeling of numerous tissues in preclinical studies and human clinical applications. The mechanisms by which ECM induces constructive remodeling are not well understood, but it appears that the degradation products of ECM scaffolds may play key roles in cell recruitment and constructive remodeling. The objective of the present study was to investigate the effects of age and species of the tissue from which ECM is harvested on the chemoattractant activity of degradation products of ECM for human keratinocyte stem and progenitor cells. Adult human skin ECM, fetal human skin ECM, and adult porcine skin ECM were prepared, enzymatically digested, characterized by SDS-PAGE, and evaluated for *in vitro* chemoattractant activity for human keratinocyte progenitor and stem cells (HEKn). Degradation products of human fetal skin ECM showed greater chemoattractant activity than human adult skin ECM degradation products for the HEKn. Degradation products of porcine adult skin ECM showed greater chemoattractant activity than human adult skin ECM. The human fetal skin ECM degradation products showed the strongest chemoattractant activity for the HEKn. The findings of this study support the concept that the mechanism of ECM scaffold remodeling involves the recruitment of lineage-directed progenitor cells by scaffold degradation products and that both the age and species of the tissue from which the ECM is harvested have an effect upon this chemoattractant potential.

Keywords

extracellular matrix; scaffold; degradation; cell recruitment; chemotaxis; keratinocyte

1. Introduction

Biologic scaffolds composed of naturally occurring extracellular matrix (ECM) have been successfully used as templates for the constructive remodeling of numerous tissues in preclinical studies and human clinical applications (Badylak et al., 1989; Badylak et al., 1995; Kropp et al., 1995; Kropp et al., 1996; Vaught et al., 1996; Dahms et al., 1997; DeJardin et al., 1999; Badylak et al., 2000; Atala 2002; Badylak et al., 2003; Badylak 2004; Badylak et

al., 2005). The mechanisms by which ECM induces constructive remodeling are largely unknown, but it appears that the degradation products of ECM scaffolds play a key role in the cell recruitment and constructive remodeling effect (Sarikaya et al., 2002; Li et al., 2004; Brennan et al., 2006; Reing et al., in press; Beattie et al., in press). ECM scaffolds that are not chemically crosslinked (e.g., carbodiimide-treated) are rapidly degraded in vivo (Badylak et al., 1998; Record et al., 2001; Gilbert et al., 2006) and have been shown to recruit bone marrow derived cells to the site of remodeling (Badylak et al., 2001; Zantop et al., 2006). These marrow-derived cells remain in the remodeling site beyond the time period of an expected inflammatory response, integrate into the remodeled tissue, and may differentiate into site-appropriate mature cell types (Zantop et al., 2006).

Most, if not all, tissues and organs within the adult human body harbor multipotential stem and progenitor cell populations that are specific for the tissue or organ within which they reside. These tissue-specific stem and progenitor cells play important roles in tissue homeostasis and repair following injury, and are found within bone marrow, muscle, adipose tissue, lungs, liver, small intestine, and skin, among other tissues (Brittan and Wright 2002; Kim et al., 2005; Vaananen 2005; Herrera et al., 2006; Moore and Lemischka 2006; Wilson and Trumpp 2006; Gimble et al., 2007; Levy et al., 2007; Mimeault et al., 2007; Peault et al., 2007; Crisan et al., in press). Human keratinocyte stem and progenitor cells have been well characterized and numerous markers have been identified for these cells, including keratinocyte stem and progenitor cell marker $\beta 1$ integrin (Jones et al., 1995; Jensen et al., 1999; Janes et al., 2002; Roh and Lyle 2006), keratinocyte stem cell markers cytokeratin 15 (CK15) (Lyle et al., 1999; Cotsarelis 2006; Roh and Lyle 2006) and cytokeratin 19 (CK19) (Janes et al., 2002; Larouche et al., 2005), as well as Rex-1, a putative human stem cell marker (Brivanlou et al., 2003; Richards et al., 2004; Mongan et al., 2006).

Because ECM produced by the resident cells of each tissue is uniquely suited with regard to composition and structure for that particular tissue, the composition of ECM harvested from different tissues will vary (Werb and Chin 1998; Brown et al., 2006; Sellaro et al., 2007). It is logical and possible that ECM derived from a particular tissue may generate degradation products that recruit progenitor cells that are lineage-directed for that same tissue. Furthermore, it is known that fetal tissue has a greater regenerative capacity, particularly in skin wounds, than adult tissue (Bullard et al., 2003). Differences between fetal and adult wound healing are likely due to differences in both the cell population and the ECM (Bullard et al., 2003).

The objective of the present study was to investigate the effects of age and species of the tissue from which ECM is harvested on the chemoattractant activity of degradation products of ECM for human keratinocyte stem and progenitor cells.

2. Materials and Methods

2.1 Preparation of ECM Degradation Products

Human fetal skin ECM (hfS-ECM) was prepared from fetal skin samples obtained from elective pregnancy termination at 18–23 weeks gestation in a protocol approved by the University of Pittsburgh IRB. Fetal skin was immersed in water at 4° C for 4–18 hours to facilitate the removal of excess connective tissue. The tissue was then placed in 0.02% trypsin (Sigma, St. Louis MO)/0.05% ethylenediaminetetraacetic acid (EDTA) (Sigma) at 37° C for 1 hour, then placed on a rocker in 3% (v/v) Triton-X (Sigma) at 4° C for 48 hours, and finally in 4% (w/v) deoxycholic acid (Sigma) at 4° C for 24 hours.

Human adult skin ECM (hS-ECM) was prepared from an adult skin sample obtained from elective surgery in a protocol approved by the University of Pittsburgh IRB. The skin was immersed in water at 4° C for 4–18 hours to facilitate the removal of excess connective tissue.

The epithelium was removed from the skin sample and the remaining tissue was then placed in 0.02% trypsin (Sigma, St. Louis MO)/0.05% ethylenediaminetetraacetic acid (EDTA) (Sigma) at 37° C for 3 hours, then placed on a rocker in 3% (v/v) Triton-X (Sigma) at 4° C for 48 hours, and finally in 4% (w/v) deoxycholic acid (Sigma) at 4° C for 24 hours. To remove adherent adipose tissue, the sample was incubated in 1-Propanol (Sigma) for three 2-hour washes on an orbital shaker at room temperature. The sample was then washed extensively in water to remove all traces of alcohol.

Porcine adult skin ECM (pS-ECM) was prepared from skin obtained from a local abattoir immediately after death of pigs weighing approximately 100–120 kg. Connective tissue and the epithelium were removed from the skin samples and the remaining tissue was placed in deionized water at 4° C for 24 hours. The tissue was then placed in 0.02% trypsin (Sigma)/0.05% ethylenediaminetetraacetic acid (EDTA) (Sigma) at 37° C for 5 hours, then placed on a rocker in 3% (v/v) Triton-X (Sigma) at 4° C for 48 hours, and finally in 4% (w/v) deoxycholic acid (Sigma) at 4° C for 48 hours.

Following these processing steps, all ECMs were treated with 0.1% peracetic acid/4% ethanol for two hours and rinsed with deionized water and phosphate buffered saline (PBS). Complete decellularization was confirmed by both 4'-6-diamidino-2-phenylindole (DAPI) nuclear staining and hematoxylin and eosin staining of 5 µm thick sections of the ECMs. The ECM sheets were then frozen and lyophilized. Lyophilized hS-ECM and pS-ECM sheets were frozen and comminuted into a particulate form using a Waring commercial blender and Wiley Mill [25]. Due to quantitative limitations for hfS-ECM material and loss of material associated with use of the Wiley Mill, hfS-ECM was manually cut into small pieces.

Each type of ECM (hfS-ECM, hS-ECM, pS-ECM) was digested using pepsin. Particulate lyophilized ECM was added to 1 mg/mL pepsin (Sigma) in 0.01 N HCl for a final concentration of 10 mg ECM/mL suspension. The suspension was mixed on a stir plate at room temperature for 48 hours, at which time no visible pieces of ECM remained. Pepsin control samples were prepared by mixing the pepsin digestion solution (1 mg/mL pepsin in 0.01 N HCl) at room temperature for 48 hours.

2.2 SDS-PAGE analysis of ECM digests

A bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL) was performed to estimate protein concentration of each type of ECM pepsin digest. Based on BCA assay results, uniform quantities of protein were loaded into each lane of a 12.5% polyacrylamide gel, with a prestained protein standard (Bio-Rad, Hercules, CA) ranging from 15 to 250 kDa. The gel was run at a voltage of 80 V until the dye front entered the separating gel, then at a constant voltage of 100 V until the dye front reached the bottom of the gel. The gel was stained with Imperial Protein Stain (Pierce Biotechnology).

2.3 Culture of HEKn

Neonatal human epidermal keratinocytes (HEKn) were obtained from Cascade Biologics and cultured in EpiLife medium (Cascade Biologics, Portland, OR) supplemented with Human Keratinocyte Growth Supplement (HKGS, Cascade Biologics), yielding final supplemented medium concentrations of 0.2% (v/v) bovine pituitary extract, 5 µg/mL bovine insulin, 0.18 µg/mL hydrocortisone, 5 µg/mL bovine transferrin, and 0.2 ng/mL human epidermal growth factor. HEKn were cultured as per product instructions. Cells used in these studies were passage four through passage seven.

2.4 Characterization of HEKn

Neonatal human epidermal keratinocytes (HEKn) were plated into CultureSlides (BD Falcon, Franklin Lakes, NJ) and cultured as described above. Cells were fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized in 0.1% Triton X-100 in PBS for 5 min. Cells were blocked in 1% bovine serum albumin and 0.1% Tween 20 in PBS for 30 min at room temperature. Cells were then incubated with primary antibodies for 1 h at room temperature and subsequently incubated with Alexa Fluor 594- and/or Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, now a part of Invitrogen) for 1 h at room temperature. The cells were washed in PBS and mounted using Vectashield mounting medium containing DAPI to counterstain nuclei (Vector Laboratories, Burlingame, CA). Cells were examined with a Nikon Eclipse TE-2000-E fluorescence microscope and the Nikon Elements Advanced Research quantitation software. For each primary antibody, cells of 9–12 fields were photographed and counted. The primary antibodies used in this study were: Rabbit anti-human Rex-1 antibody raised in our laboratory (1:10 dilution); mouse monoclonal anti-human CK15 and CK19 (1:200 dilution) purchased from Novocastra (New Castle, UK); and mouse monoclonal anti-human β 1 integrin (1:200 dilution) purchased from Chemicon (now a part of Millipore). Reported results are expressed as the mean and standard error of the percentage of cells staining positive for each antibody.

2.5 Chemotaxis assay

In vitro chemotaxis of HEKn toward ECM pepsin digests was evaluated using a 48-well micro chemotaxis chamber (Neuro Probe, Gaithersburg, MD). When HEKn reached 70–80% confluency, they were starved overnight in unsupplemented EpiLife medium before use in chemotaxis assays the following morning. Then, HEKn were trypsinized, neutralized, and centrifuged as per HEKn product instructions, resuspended in unsupplemented EpiLife medium, and incubated in suspension for one hour at 37°C.

ECM pepsin digest samples were diluted in unsupplemented EpiLife medium to concentrations of 100, 200, 500, and 1000 μ g ECM digest/mL. A negative control for each sample was prepared by diluting the pepsin control sample in unsupplemented EpiLife medium at the same ratios as the ECM pepsin digest samples. A positive control of 10% HKGS in unsupplemented EpiLife medium was also included in each assay. Samples were loaded to the lower chamber of each well of the chemotaxis chamber. An 8 μ m pore size polycarbonate filter coated with collagen I (BD Biosciences, San Jose, CA) divided the lower and upper chamber of each well. Approximately 30,000 cells were added to the upper chamber of each well. The chemotaxis chamber was incubated at 37°C for 3 hours. Cells that had not migrated through the pores of the filter were removed and migrated cells were fixed, permeabilized, and stained with DAPI.

Three random fields from each well, located in the upper left region of each well, upper right region of each well, and lower center of each well, were photographed at 200 $\times$ magnification and migrated cells were counted from these fields using ImageJ software. Samples were tested in quadruplicate for each chemotaxis assay performed. The percent change in migrated cells in response to ECM pepsin digest samples compared to negative control samples was calculated for each assay performed. To compare results between different assays, the percent change was normalized by the percent change induced by the positive control for each assay. Reported results are expressed as the mean and standard error of percent change in migration normalized by positive control from four assays (n=4 independent assays, with samples run in quadruplicate, for n=16 samples tested). Statistical significance between samples was determined by Student's t-test.

3. Results

SDS-PAGE analysis of hfS-ECM, hS-ECM, and pS-ECM digests (Figure 1) showed that all four ECM digests contained numerous peptides ranging in size from 15 to 250 kD. Certain peptides appeared to be present in all four ECM digests, while others were only present in one type of ECM digest, suggesting that there were variations in molecules released by pepsin digestion of the different skin ECMs. Bands that were present in all four ECM digests showed different intensity suggesting quantitative differences between the amounts of the respective molecules.

HEKn in culture were found to express human Rex-1 (Brivanlou et al., 2003; Richards et al., 2004; Mongan et al., 2006), CK15 (Lyle et al., 1999; Cotsarelis 2006; Roh and Lyle 2006), and CK19 (Janes et al., 2002; Larouche et al., 2005) mRNA (data not shown), markers known to be present in keratinocyte stem cell populations. Fluorescent immunolabeling demonstrated that Rex-1 signal was present in both the cytoplasm and the nucleus of HEKn, and the Rex-1 signal in the nucleus was greater than that in the cytoplasm (Figure 2a). CK15 and CK19 signals were detected in the cytoplasm of HEKn (Figure 2b and 2c). β 1 integrin, a cell surface marker found on both skin stem cells and progenitor cells (Jones et al., 1995; Jensen et al., 1999; Janes et al., 2002; Roh and Lyle 2006), was present in the cytoplasmic membrane of HEKn (Figure 2d). Quantification of the fluorescently immunolabeled cell populations showed that 100% of HEKn stained positively for β 1 integrin (Figure 3). Approximately 26% of HEKn stained positively for Rex-1 and CK15 and approximately 12% of HEKn stained positively for CK19 (Figure 3). CK15 and CK19 were often co-localized with Rex-1. These results indicate that the HEKn population used in chemotaxis assays consisted of approximately 26% keratinocyte stem cells, and that the remainder were more differentiated skin progenitor cells.

Pepsin digested hfS-ECM showed strong, dose dependent chemoattractant activity for HEKn. A greater than 500% increase in HEKn migration was observed at 1000 μ g/mL hfS-ECM pepsin digest compared to the negative control (Table 1).

Pepsin digested hfS-ECM showed significantly stronger chemoattractant activity for HEKn than both hS-ECM and pS-ECM pepsin digests at 100, 200, 500, and 1000 μ g/mL ($p < 0.05$, Figure 4). pS-ECM pepsin digest showed significantly stronger chemoattractant activity than hS-ECM pepsin digest at 200, 500, and 1000 μ g/mL ($p < 0.05$, Figure 4).

Migrated and fixed HEKn on chemotaxis chamber filters were fluorescently immunolabeled for Rex-1, CK15, and CK19. Nearly all HEKn that had migrated in response to hfS-ECM pepsin digest stained positive for Rex-1, CK15, and CK19, suggesting that hfS-ECM was particularly chemoattractant for keratinocyte stem cells compared to keratinocyte progenitor cells, selecting in particular for the CK19-positive subset of keratinocyte stem cells (Figure 5).

4. Discussion

The present study demonstrates that degradation products of human fetal skin-derived ECM possess stronger chemoattractant activity for skin-specific lineage-directed stem and progenitor cells than do degradation products of human adult skin-derived ECM. In addition, degradation products of porcine adult skin-derived ECM showed stronger chemoattractant activity than degradation products of human adult skin-derived ECM. These results suggest that ECM degradation products from younger tissue sources may have more potent chemoattractant activity for local tissue stem or progenitor cells than older tissue sources, and that the species of the tissue source also has an effect on chemoattractant activity.

The early gestation human fetus (less than 24 weeks gestation), unlike the later-gestation fetus or adult, is able to heal incisional skin wounds without scarring (Adzick 1992). This scarless

wound healing ability of fetal skin may be due to the fetal cells and/or the fetal ECM. Fetal platelets, inflammatory cells, and fibroblasts all demonstrate differences from their adult counterparts that may contribute to the scarless wound healing phenomenon. Fetal ECM contains a higher proportion of type III collagen and a greater concentration of hyaluronic acid than ECM of adult skin. Unwounded fetal skin at gestational ages associated with scarless wound healing has also been shown to express low levels of TGF β 1, high levels of TGF β 3, and increased expression of matrix metalloproteinases, a family of proteases associated with ECM degradation and remodeling (Bullard et al., 2003). Although the present study does not address the relative contribution of cells vs. ECM to scarless fetal wound healing, it appears clear that the ECM, which represents the secreted product of local cells, contains signaling molecules that can affect stem and progenitor cell activity.

ECM scaffolds implanted *in vivo* in preclinical studies have been shown to result in constructive remodeling, promote angiogenesis, and to resist bacterial infection (Badylak et al., 1994; Badylak 2002; Badylak et al., 2003; Li et al., 2004; Shell et al., 2005). *In vitro* studies have shown that degradation of ECM derived from several tissues generates low molecular weight peptides with biological properties such as chemotactic, angiogenic, and antimicrobial activity (Sarıkaya et al., 2002; Li et al., 2004; Brennan et al., 2006). In contrast, intact ECM does not possess such activity (Holtom et al., 2004), suggesting that these biological activities are associated with the products of ECM degradation, rather than molecules present in intact ECM. Low molecular weight peptides isolated from acid-hydrolyzed small intestinal submucosa (SIS-ECM) have been shown to possess chemotactic activity for primary murine adult liver, heart, and kidney endothelial cells and to promote vascularization *in vivo* in Matrigel plug assays (Li et al., 2004). A recent study has shown that *in vivo* degradation of urinary bladder-derived ECM can produce bioactive matricryptic peptides that cause chemotaxis of multi-potential progenitor cells (Beattie et al., in press). Previous studies have also shown that subcutaneous implantation of porcine SIS-ECM induces migration of bone marrow-derived cells to the site of constructive remodeling (Badylak et al., 2001), and that this phenomenon is associated with complete degradation of the ECM scaffold. The present study adds a new perspective to the role of the ECM in response to injury; specifically, that local, tissue-specific lineage-directed progenitor and stem cells may be selectively recruited to the appropriate site.

In the context of regenerative medicine, the findings of the present study support the concept of progenitor cell recruitment associated with ECM scaffold remodeling and its potential upon constructive remodeling of tissues. The findings also suggest that the chemoattractant activity of ECM degradation products for progenitor and stem cells decreases as the age of the tissue from which the ECM is harvested increases. Given the known phenomenon of scarless fetal wound healing (Bullard et al., 2003), and greater capacity for wound healing in young children compared to adults (Illingworth 1974), these results are not surprising. The apparent greater chemotactic activity of adult porcine vs. human tissue may be the result of the relative age of the two “adult” species. Six to seven month old pigs are considered mature but are unlikely to be as “aged” as adult humans that can by definition be any number of years older than twenty-one. A more comprehensive study with different ages of animals would be necessary to fully address this issue.

The concept of functional matricryptic peptides is not new. During ECM degradation, many large insoluble molecules present within the matrix are reduced into fragments which possess biological activities that are not possessed by the parent molecules. In addition to proteolysis-generated bioactive fragments, functional sites of the parent molecules that are hidden and inactive within the ECM can also become active due to conformational changes (Schenk and Quaranta 2003). Some of the ECM molecules that have been shown to possess this property are collagen, (Ramchandran et al., 1999; Xu et al., 2001; Xu et al., 2001; Ortega and Werb 2002; Mott and Werb 2004), fibronectin (Ambesi et al., 2005), laminin (Ponce et al., 2003;

Schenk and Quaranta 2003), and hyaluronan (Deed et al., 1997; Chen and Abatangelo 1999; Slevin et al., 2002), all of which are present in ECM scaffold materials (Badylak 2002).

It is important to note that the chemoattractant activity of the ECM digests evaluated in the present study represent the net activity of bioactive molecules present within each of the digests. This net effect of chemoattractant activity is logical and relevant from an *in vivo* injury repair perspective. It is unclear if the concentrations of ECM digests evaluated in the present study are the same as the concentrations that occur *in vivo* during the process of ECM degradation, and if the chemoattractant molecules released by pepsin digestion of ECM would also be released during *in vivo* degradation. The specific peptides/molecules responsible for these chemoattractant effects have not yet been identified and clearly this is an important next step.

The results of this study suggest that degradation products of ECM possess chemoattractant activity for local tissue progenitor and stem cells, and that this chemoattractant activity may decline as a function of the age of the tissue from which the ECM is harvested, and that ECM may vary between different species. These findings add a new perspective to the role of ECM in wound healing and the differences between fetal and adult wound healing. Though fetal ECM is not a likely candidate for tissue engineering/regenerative medicine applications because of the scarce availability of the raw material, the findings of the present study could be applied to the development of methods to induce migration of lineage-directed progenitor cells to tissue sites in need of repair, thereby facilitating a regenerative tissue response rather than default scar tissue formation.

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Figure 1.
Pepsin digests of human fetal skin ECM (hfS), human adult skin ECM (hS), and porcine adult skin ECM (pS) were separated on 12.5% SDS-PAGE. Protein bands were visualized by Imperial Protein Stain and photographed.

Figure 2.

(A) Fluorescent immunolabeling of cultured HEK cells for Rex-1. (B) Fluorescent immunolabeling of cultured HEK cells for CK15. (C) Fluorescent immunolabeling of cultured HEK cells for CK19. (D) Fluorescent immunolabeling of cultured HEK cells for $\beta 1$ integrin.

Figure 3.

Quantification of HEKn staining positive for Rex-1, CK15, CK19, and β 1 integrin. Mean value $\pm$ standard error (n=9–12). No error bars are shown for β 1 integrin because 100% of HEKn stained positive in all photographed fields.

Figure 4. Chemoattractant activity of pepsin digests of hfS-ECM, hS-ECM, and pS-ECM for HEK cells. Mean value $\pm$ standard error (n=4), * = $p < 0.05$ versus hS-ECM, # = $p < 0.05$ versus pS-ECM.

Figure 5.
Quantification of migrated HEK cells staining positive for Rex-1, CK15, and CK19. Mean value $\pm$ standard error (n=5–6).

Summary of data from one representative HEK293T chemotaxis assay, including number of migrated cells for each sample tested in quadruplicate, mean of migrated cells, standard error of the mean, p-value from a t-test comparing each sample to its negative control, and calculated percent change in migrated cells for each sample compared to its negative control

Table 1

Sample	Migrated Cells				Mean	Standard Error	p-value	Negative Control
10% HKGS	133	146	112	100	123	10.3	0.000	485
EpiLife medium	29	19	22	14	21	3.1	--	--
100 µg/mL hFS-ECM digest	19	18	27	30	24	3.0	0.019	68
Pepsin control for 100 µg/mL hFS-ECM digest	8	15	16	17	14	2.0	--	--
200 µg/mL hFS-ECM digest	33	39	22	18	28	4.8	0.016	96
Pepsin control for 200 µg/mL hFS-ECM digest	14	14	17	12	14	1.0	--	--
500 µg/mL hFS-ECM digest	26	49	28	30	33	5.3	0.001	393
Pepsin control for 500 µg/mL hFS-ECM digest	6	6	8	7	7	0.5	--	--
1000 µg/mL hFS-ECM digest	35	39	19	35	32	4.4	0.000	540
Pepsin control for 1000 µg/mL hFS-ECM digest	6	4	6	4	5	0.6	--	--