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Stimulation of *In Vivo* Angiogenesis Using Dual Growth Factor-loaded Crosslinked Glycosaminoglycan Hydrogels

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Abstract

Crosslinked, chemically modified hyaluronan (HA) hydrogels preloaded with two cytokine growth factors, vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1), were employed to elicit new microvessel growth *in vivo*, in both the presence and absence of heparin in the gels. HA hydrogel film samples were surgically implanted in the ear pinnae of mice, and the ears were harvested at 7 or 14 days post implantation. Analysis of neovascularization showed that each of the treatment groups receiving an implant, except for HA/Hp at day 14, demonstrated significantly more microvessel density than control ears undergoing surgery but receiving no implant ($p < 0.015$). Treatment groups receiving either Ang-1 alone, or aqueous co-delivery of both Ang-1 and VEGF, were statistically unchanged with time. In contrast, film delivery of both growth factors produced continuing increases in vascularization from day 7 to day 14 in the absence of heparin, but decreases in its presence. However, presentation of both VEGF and Ang-1 in crosslinked HA gels containing heparin generated intact microvessel beds with well defined borders. The HA hydrogels containing Ang-1+VEGF produced the greatest angiogenic response of any treatment group tested at day 14 ($NI = 7.44$ in the absence of heparin and 4.67 in its presence, where NI is a neovascularization index). Even in the presence of heparin, this was 29% greater vessel density than the next largest treatment group, that receiving HA/Hp+VEGF ($NI = 3.61$, $p = 0.04$). New therapeutic approaches for numerous pathologies could be notably enhanced by the localized, sustained angiogenic response produced by release of both VEGF and Ang-1 from crosslinked HA films.

Keywords

Angiogenesis; Controlled Drug Release; Glycosaminoglycans; Growth factors; Cytokine; Vascular Endothelial Growth Factor; Angiopoietin-1

1. INTRODUCTION

Recent approaches for therapeutic tissue regeneration, of bone or blood vessels for example, usually involve one-time delivery of single growth factors. In such schemes, a single cytokine is administered either as a liquid aliquot or within a solid implant, delivered simultaneously with the engineered tissue to be implanted. It has been shown that angiogenesis can be achieved

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through therapeutic cytokine delivery by many investigators [1–3]. However, long term viability and functionality of the blood vessels formed has not been demonstrated [4].

One important reason for the lack of greater clinical success in creating viable capillary networks appears to be the intricacy of the process of angiogenesis. Formation of new capillary vessels from existing microvessels is initiated by peptide growth factors. Subsequent release of proteinase enzymes leads to degradation of the extracellular matrix (ECM) and parent capillary basement membrane, liberating ECM-bound growth factors such as vascular endothelial growth factor (VEGF) [5]. Endothelial cells are then able to proliferate into the extracellular space and form new capillary sprouts. The sprouts continue to grow by endothelial proliferation, and a lumen eventually forms as the sprouts organize into tubules. New vessels reach maturity when contiguous vessels connect and a new basement membrane is formed [6,7]. This process of capillary formation is a central aspect of many physiologic functions, including tissue and organ growth, wound healing, female reproductive function, as well as pathologic tumor formation.

The stages of angiogenesis are known to be regulated by over two dozen stimulatory or inhibitory cytokine growth factors that act at various times and in complex ways [6–10]. Of these growth factors, the most important as well as most studied is VEGF. Furthermore, various ECM glycoproteins, glycosaminoglycans (GAGs) and proteins, including collagen, have also been shown to have regulatory effects on microvessel growth [11,12]. The cascade of events surrounding the development of mature microvessel networks depends on interactions between all these factors. Consequently, one possible way to enhance the angiogenic response to exogenously delivered biomaterials would be to introduce more than one growth factor with the implant.

The development of chemically modified forms of hyaluronic acid (HA) that are able to form biocompatible, macroporous hydrogels opens new ways to store and release combinations of growth factors *in vivo* [13–15]. HA is the only non-sulfated GAG in the ECM of vertebrates. It consists of repeating disaccharide units (β -1,4-D glucuronic acid— β -1,3- N acetyl-D glucosamine), with an overall molecular weight between 100 and 5000 kDa [16]. HA is a strong inducer of angiogenesis, although this phenomenon depends on molecular weight [17–19]. High molecular weight HA (*n*-HA) has been shown to inhibit angiogenesis [20], but low molecular weight HA (*o*-HA) has the opposite effect, stimulating endothelial cell proliferation and migration [21–23]. Therefore, when HA is used as a delivery vehicle for growth factors, it can be expected to actively participate in tissue responses, rather than serving only as an inert conduit. For example, Peattie *et al.* showed in a mouse ear pinna study that delivery of VEGF in HA-based hydrogels produced an angiogenic effect greater than the sum of the effects of HA and VEGF delivered singly [15]. However, although the resulting endothelial proliferation was substantial, mature functional microvessels were not observed.

Our group recently reported an initial study of *in vivo* delivery of two cytokines, under the hypothesis that new microvessel function can be improved by simultaneous delivery of one growth factor chosen to stimulate an initial angiogenic response and a second selected for later vessel maturation [24]. These implants resulted in microvessels organized into recognizable tubular networks filled with erythrocytes. Recently it has also been shown that heparin (Hp), a highly sulfated glycosaminoglycan known primarily for its antithrombotic function, has high affinity for basic growth factors and is capable of sequestering those GFs in the ECM. In correspondence with that function, we anticipate that heparin covalently immobilized within HA-based gels can both chemically and functionally mimic the heparan sulfate proteoglycans (HSPGs) normally present in the ECM. Accordingly, in the present study we hypothesize that inclusion of small amounts of Hp in HA-based gels can regulate *in vivo* growth factor release and thereby produce mature, more functional microvessel networks. To test that hypothesis,

implants containing VEGF along with angiopoietin 1 (Ang-1) were delivered in a mouse model. We show that the incorporation of heparin leads to more defined microvessels and fewer extravasated cells as compared to controls lacking Hp.

2. MATERIALS AND METHODS

2.1 Materials

Fermentation-derived HA (sodium salt, $M_W = 750$ kDa) was a gift from Novozymes Biopolymers A/S (Bagsvaerd, Denmark). Heparin, (sodium salt from porcine mucosa, unfractionated, $M_W = 15$ kDa), vascular endothelial growth factor (human recombinant, 165 amino acids, $M_W = 46$ kDa) and angiopoietin-1 (human recombinant, 476 amino acids, $M_W = 66$ kDa) were purchased from Sigma Chemical Co. (St. Louis, MO). Poly(ethylene glycol)-diacrylate (PEGDA, $M_W = 3400$ Da) was obtained from Nektar Therapeutics (Huntsville, AL). Male Balb/C mice were provided by Simonson Laboratories (Gilroy, CA).

2.2 Hydrogel film preparation

HA-DTPH and Hp-DTPH, the thiol-modified forms of hyaluronic acid and heparin, were synthesized as previously described [25–27]. In brief, HA (or Hp) was modified with 3,3 dithiobis(propanoic hydrazide) (DTP) in the presence of EDCI, then reduced with dithiothreitol (DTT), resulting in HA-DTPH after dialysis. Non-heparinized glycosaminoglycan hydrogels were prepared by dissolving HA-DTPH in deionized H_2O at a concentration of 12.5 mg/ml, maintaining solution pH at 7.4 by dropwise addition of K_2HPO_4/KH_2PO_4 0.1 M and 0.01 M as needed. Heparinized cases were prepared similarly, with 12.4625 mg/ml HA-DTPH and 0.0375 mg/ml Hp-DTPH (0.3% Hp w/w relative to HA-DTPH). PEGDA was also dissolved in deionized H_2O , at a concentration of 91 mg/ml, and the pH was maintained at 7.4. Hydrogels were then formed with PEGDA as crosslinking agent for the HA-DTPH chains by adding volumes of each solution to a small, polystyrene dish at a 1:2 ratio of acrylate to thiol functionalities, at room temperature, with gentle swirling. Although crosslinking proceeded rapidly, within 20–30 minutes, to ensure fully crosslinked, homogeneous gels the solution was gently agitated on an orbital platform for 24 hours. The resulting gels were then placed in a 37°C incubator for 24 hours, or until the gels were thoroughly dehydrated to durable, flexible, 1mm thick films. Films were stored in a dark enclosure at room temperature until needed. Round discs of 4 mm diameter, containing 0.25 mg HA-DTPH, were cut from these films at the time of surgical implantation.

In the hydrated state, these HA-DTPH hydrogels have been shown to take on an open, porous structure suitable for storing molecules within the gel [26]. Therefore, to incorporate growth factors in the gel, liquid aliquots of VEGF, Ang-1, or both were non-covalently mixed with the dissolved HA-DTPH (and Hp-DTPH when applicable) before crosslinking. Experiments were performed with concentrations that resulted in 25 ng of each growth factor delivered to the animal within the 4-mm film disc for non-heparinized gels. However, 100 ng of each was delivered in heparin-containing disks, to compensate for the slower anticipated release.

2.3 Surgical and experimental procedures

The ability of growth factor-loaded HA-DTPH hydrogels to stimulate angiogenesis was tested on live animal models. All procedures were carried out with the full approval of the Oregon State University Institutional Animal Care and Use Committee. Male Balb/C mice aged 6–8 weeks were anesthetized using 2.5% isoflurane with an inhalation anesthesia system (Summit Medical Equipment, Inc., Bend, OR). Once a deep general anesthetic plane had been reached, a shallow 4–5mm incision was created through the superficial skin on the posterior pinna of the right ear. A blunt probe was inserted through the incision, and a 5-mm pocket created under the skin. If the animal was to receive an implant, a 4-mm HA-DTPH disc was placed inside

the pocket, and the incision was closed without sutures. If the animal was to receive a liquid growth factor aliquot, the volume of liquid was delivered to the pocket by pipette. Mice recovered without incident within 5–10 minutes after these brief surgeries, and the incisions healed in 5–7 days.

On days 7 or 14 post implantation, the mice were anesthetized by overdose of isoflurane (5%) and sacrificed by cervical dislocation. Both the surgical (right) and contralateral ears were retrieved and fixed in formalin. The ears were then embedded in paraffin, thin sectioned parallel to the pinna surface, and stained with hemotoxylin and eosin (H&E). Microvessels were counted directly from the slides at 400× magnification after identification marks on each slide were covered so the observer was blind to which treatment group the slide belonged. Ten locations per ear from within the pocket area, were selected at random for quantification resulting in microvessel density representative of that ear.

2.4 Data analysis

A total of 6 animals ($n = 6$) received implants for each treatment case and each time point. Microvessel density data are accordingly presented as mean $\pm$ standard deviation. Due to the number of observers in our laboratory a protocol has been developed to minimize the effects of intra-observer variability. For that purpose, a set of 22 slides from 11 treatment cases were set aside and vessels in them counted by each member of the laboratory. Group averages were then calculated, and a correction factor determined for each individual observer to equate that individual's counts to the group means. This correction procedure has been applied to all the data presented in this paper. The vessel counts presented here were performed independently by two observers.

To account for the number of vessels present before surgery as well as those induced by the surgical procedure alone, so that the effect of the gel and/or growth factor addition could be identified, a dimensionless *Neovascularization Index* (NI) was defined as,

$$NI = \frac{(\overline{treatment - CL}) - (\overline{sham - CL})}{\text{mean } CL} \quad (1)$$

where $(\overline{treatment - CL})$ refers to the vessel count from the implanted ear of a particular animal minus that of its contralateral ear, averaged over all the animals in a particular treatment group, $(\overline{sham - CL})$ represents the same quantity for a sham surgery control case that underwent pocket formation but received no implant, and $\text{mean } CL$ is the average count from all contralateral ears over all treatment groups. Thus defined, NI represents the number of additional vessels present post-implant in a treatment group, minus the additional number due to the surgical procedure alone, normalized by the average contralateral count [15]. (Mathematically, NI can be expected to be a Gaussian random variable, since both $(treatment - CL)$ and $(sham - CL)$ are themselves random variables. Calculation of its mean and standard deviation therefore requires appropriately linearly combining the properties of the constituent terms.)

Statistical significance was determined using a two-way ANOVA and post hoc Fisher's PLSD analysis (StatView 5.0, SAS Institute Inc., Cary, NC), with significance taken at the level $p \leq 0.05$.

3. RESULTS

Hydrogel films for this study were prepared with thiolated derivatives of HA and Hp. These disulfide-crosslinked hydrogel films are stable and highly bio- and cytocompatible both *in vitro* and *in vivo* [25–28]. In addition, their physico-chemical properties of mechanical strength,

ability to degrade *in vivo* without systemic sequelae and favorable interactions with VEGF and other important growth factors [15] make them ideally suited for delivery of bioactive agents. Prior to crosslinking, the degree of substitution on the HA or HA/Hp was found to be 42%, based on the number of available glucuronate residues modified [25]. Gels formed quickly by the addition crosslinking reaction of thiols to PEGDA at a neutral to slightly basic pH (Figure 1). Subsequently these gels can be dehydrated to a film state, which swells reversibly with no degradation when rehydrated in an aqueous solution [26].

In vivo microvessel development was assessed in an ear pinna model for a series of control and experimental conditions, in both the presence and absence of heparin. Non-heparinized cases included (i) creation of a pocket with no implant placed, (ii) Ang-1 alone, delivered as a liquid aliquot so that its physiologic effects would not be influenced by interaction with a hydrogel, (iii) a combined Ang-1+VEGF liquid aliquot, (iv) an HA film preloaded with Ang-1, and (v) an HA film preloaded with both VEGF and Ang-1. Heparinized cases included (vi) an HA film containing only Hp with no growth factors, (vii) an HA/Hp film preloaded with Ang-1, (viii) an HA/Hp film preloaded with VEGF, and (ix) an HA/Hp film containing both Ang-1 and VEGF. Whenever possible, results for control cases were retained from previous experiments [15,24]. These included (x) an HA film alone with neither Hp nor growth factors, (xi) VEGF alone delivered as a liquid aliquot, and (xii) an HA film preloaded with only VEGF. The control experiments (i-iv, vi-viii, x-xii) provided quantitative evaluation of vessel formation secondary to establishment of a surgical pocket without implant placement, as well as of the tissue response to growth factors or film individually. Therefore, they allowed direct evaluation of the effects attributed to implanted films preloaded with both cytokines (v, ix).

Representative photographic images of tissue microvascularization show significant differences that characterized the tissue response to each implant (Figure 2). Contralateral ear sections (Figure 2a) had a similar appearance for all animals. No evidence of any systemic response to the implant was observable at any time point for any of the treatment cases. Chondrocytes were widely distributed in this tissue, along with numerous hair follicles and sebaceous and other glands. Few capillaries were evident, but the endothelial borders of those that were present were well-defined and intact. Red cells were confined within fully developed capillary walls.

Ears implanted with HA+Ang-1+VEGF disks (Figure 2b) continued to show the chondrocytes, hair follicles and glands typical of ear pinnae, but showed distinct histological differences from the contralateral controls. Although many apparently partially formed new microvessels could be recognized in these ears through their associated chains of red cells, the endothelial borders of these vessels were poorly developed and incomplete. Accordingly, large numbers of extravasated erythrocytes could be found distributed among the identifiable vessels. In addition, polymorphonuclear leukocyte inflammatory cells were also identifiable in these sections. Such leukocytes were recognizable by their multilobed nuclei.

The greatest density of microvessels was found for the case HA/Hp+Ang-1+VEGF, at day 7 (Figure 2c). However, these vessels were the least fully developed of any treatment group. Although much of the tissue in these ears appeared undisturbed, regions containing very high densities of extravasated erythrocytes were also distributed through the tissue sections. There were apparently normal distributions of non-inflammatory cells, though as would be expected for tissue recovering from surgical manipulation, tissue regions with polymorphonuclear inflammatory cells could also be found.

In contrast to the highly permeable, incomplete vessels present at day 7, however, by day 14 the vessel walls for this case were more mature and much less permeable (Figure 2d). Vessel density remained high, but far fewer extravasated red cells were apparent. Instead, chains of

erythrocytes were present, and appeared to be confined within microvessels showing well developed borders and obvious endothelial cell nuclei. This ultrastructural organization suggests that the presence of heparin in the gel preparation led to a continuing development process that led to more fully formed levels of vascular maturity than implants either lacking heparin or at earlier times in the presence of heparin.

Neovessel density was quantitatively unchanged at either time in the contralateral ears of all treatment groups, indicating that the tissue response to the implants was localized to the region of the implant, with no systemic effects. Quantitative counts of microvessel density in each treatment group at 7 and 14 days post-implant are presented in Figures 3 and 4. In both the presence and absence of heparin, the greatest microvessel density was always observed for the case HA+Ang-1+VEGF. For example, in the absence of heparin at day 7 (Figure 3a), all cases showed significantly greater vessel growth than did the sham surgery ($p < 0.01$), though there were no statistically significant differences between the cases aq. Ang-1, aq. Ang-1+VEGF and HA+Ang-1. However, the case HA+Ang-1+VEGF (685.9 microvessels/mm²) elicited more vessels than did any other treatment case, 19% greater than the next largest case, HA +Ang-1 (553.5 microvessels/mm²). Similar results were found in the absence of heparin at day 14 (Figure 3b), with all cases producing many more vessels than did the sham surgery, and the statistically significantly greatest number of vessels produced by delivery of HA+Ang-1 +VEGF (768.3 microvessels/mm²; $p < 0.015$ compared to each other group). This was 21% greater than HA+Ang-1, the next greatest case again at day 14, and 10.7% greater than its value at day 7.

Trends in microvessel density for implants containing heparin were similar to those absent Hp, although vessel counts at day 7 (Figure 4a) were much higher than the corresponding counts in the absence of heparin. Much of this difference may be related to the difficulty in performing accurate counts with slides showing so many extravasated RBCs, since the vessel counts recovered to a range consistent with those of other treatment groups by day 14 (Figure 4b). Once again, on both days 7 and 14 the greatest number of vessels was observed for HA/Hp film implants containing both VEGF and Ang-1 (580.2 microvessels/mm² on day 14). This was 19.7% greater vessel density than in the next largest treatment group, that receiving HA +VEGF (466.0 microvessels/mm², although $p = 0.07$ for this comparison).

4. DISCUSSION

The ability of dual cytokine pre-loaded HA hydrogel film implants to elicit the growth and development of new capillary networks *in vivo* by providing sustained, localized growth factor delivery is well demonstrated by this experiment. The combined effects of the growth factors, in conjunction with the ability of HA and the growth factors to potentiate each other's activity [15,24], led to a strong angiogenic response even when the growth factors were delivered in very low ng doses. Furthermore, heparin-regulated delivery of two growth factors chosen to stimulate different stages of microvessel growth produced neovessel beds capable of supporting perfusion without inappropriate permeability or extravasation of red cells by two weeks post-implantation.

The microvessel density data shown in Figures 3 and 4 indicate that at both time points of the experiment, significant growth of new microvessels occurred in response to surgical intervention regardless of the type of implant placed. In the sham surgical group, vessel density at day 14 was notably decreased from its value at day 7, as would be expected for tissue recovering from perturbation. In contrast, in the absence of heparin, microvessel density for the other treatment groups was statistically unchanged from day 7 to day 14. In the presence of heparin, the very large values found on day 7 were greatly reduced on day 14 and the vessel ultrastructure was much more mature.

To distinguish the tissue response to different implants from the response to surgical intervention, vessel growth was re-expressed through the Neovascularization Index (Figures 5 and 6). In spite of the general similarity between treatment cases seemingly apparent in Figures 3 and 4, analysis with *NI* shows distinct differences in the tissue response to the different implants. In the absence of heparin (Figure 5), vessel growth was least for aqueous co-delivery of VEGF and Ang-1 ($NI = 2.83$ at day 7 and 2.26 at day 14), and greatest for delivery of both factors together in HA films ($NI = 7.44$ at day 14, $p < 0.001$ for comparisons with each other group). This difference represents a 3.3-fold increase from the least effective to the most effective treatment groups. Similarly, the most effective treatment group in the presence of heparin (Figure 6) was film delivery of both GFs ($NI = 4.67$ at day 14), for which the mean value of *NI* was 29% greater than each case delivering a single GF ($p = 0.04$ for comparison with groups containing a single growth factor, and < 0.001 for comparison with the group lacking a GF).

These data show that incorporation of small amounts of heparin, here 0.3%, in HA-based film implants significantly modifies growth factor release from the implants and the consequent microvessel growth and development. Although the values of *NI* observed on day 14 were less in the presence of Hp than in its absence, the vessel networks produced were more mature, with intact endothelial borders. In contrast, in the absence of heparin, although the microvessel networks were more dense, their borders were poorly formed and not mature (Figure 2b). Data from our previous studies using gels that did not contain heparin [24] were consistent with these trends between groups. For example, in studies with keratinocyte growth factor (KGF), HA+VEGF+KGF gels led ultimately to greater microvessel growth than any other control treatment group, including gels delivering either only VEGF or only KGF as a single GF [24]. This was also the case for both heparin-containing and non-heparin-containing gels delivering VEGF or basic fibroblast growth factor (bFGF) [29].

The fact that *NI* measurements showed a stronger angiogenic response to co-delivery of both VEGF and Ang-1 in HA-based gels than to aqueous or film delivery of either growth factor alone is consistent with the expectation that each GF has a unique affinity for heparin. As a result, each would be expected to have a unique rate of release from heparin-containing gels and consequent biologic effect. Although known primarily for its anticoagulant activity, heparin is also able to bind specific cytokines that contribute to angiogenesis. Covalent binding of growth factors such as VEGF, bFGF and transforming growth factor- β (TGF- β) is mediated by interactions between sulfate groups of Hp and arginine and lysine residues of the growth factors [30–33]. Those growth factors are thereby stored in the ECM, where local paracrine activity can occur on their release. Binding of growth factors with heparin has been shown to increase mitogenic activity, as well as enhance stability against both thermal and enzymatic denaturation [32]. Further, HA-based hydrogels can be designed to provide the same effects of protection of growth factors from proteolytic degradation and spatiotemporal control of release imparted by heparin and HSPGs in the native ECM through a covalently incorporated heparin component.

These HA-based hydrogels are fully broken down *in vivo* by the action of tissue-resident hyaluronidase after 14 days. During that period, growth factors are released from the gels by a combination of diffusive transport across the gel surface and gel degradation. It can be hypothesized that through covalent interactions with the GFs, the presence of heparin in the gel alters the mechanism of release from a diffusion-dominated process to one limited by the rate of gel breakdown. Separate studies have shown that the vascularization response elicited by growth factors when heparin is present in the gel can persist for several weeks post-implantation [29]. Such a continuing response would be expected if heparin-GF complexes remained in the local ECM at the implant site after the gel in which they were initially delivered

was fully broken down. The resulting continued presence of the growth factor would then be expected to be associated with a maintained continuation of neovessel growth.

Although covalent incorporation of modified heparin in the gels leads to controllable regulation of growth factor release, high levels of heparin have three potential adverse attributes. First, the consequent changes in the ionic character of the gel can alter buffer exchange and inhibit protein diffusion. Second, levels of heparin greater than a few percent slow the rate of gel crosslinking and decrease the gel stability. Finally, it is possible for high levels of heparin to increase the gel avidity for growth factors to a point at which release is significantly inhibited or even partially prevented. These factors make it important to minimize the gel heparin content. Previous investigations [29] showed little difference in growth factor release rates between gels containing 0 and 0.03% heparin. However, the rate of release was effectively regulated by 0.3% Hp, which produced essentially the same release rates as 3% Hp. Accordingly, to ensure that GF release would be effectively regulated but the bioactivity of released GFs maintained, we have selected 0.3% as the preferred heparin concentration for *in vivo* experiments.

Both the molecular mechanisms by which HA films induce angiogenesis and the mechanisms of interaction between HA, VEGF and Ang-1 are as yet unknown. HA has been shown to be able to potentiate the effects of VEGF both *in vitro* and *in vivo* [15,34], although it does not enable all growth factors. In particular, interactions between HA and bFGF show no similar favorable effects. *In vivo* studies with hydrogels based on adipic dihydrazide derivatives of HA found a maximal synergy for a delivered VEGF dose of 25 ng, with a lesser response that was dose-independent for greater or lesser GF loads [15].

VEGF is the only known cytokine with mitogenic effects confined to endothelial cells, and is known to be a major promoter of both physiologic and pathologic angiogenesis. Its expression correlates with capillary development during embryologic growth, wound healing and the female reproductive cycle, as well as with tumor expansion. Since *o*-HA-induced cell proliferation in monolayer cultures is not enhanced by co-addition of VEGF [34], their combined physiologic effect is not attributable to direct stimulation of endothelial cell propagation. It is possible that *o*-HA and VEGF may initiate complementary intracellular signaling pathways, or that either molecule might up-regulate receptors for the other. Moreover, during wound healing of acute partial- and full-thickness wounds, HA promotes cell movement in early granulation tissue [14,35]. This would seem to be complemented by the ability of VEGF to enhance tissue secretion of pro-angiogenic proteases including uPA, MMP-1 and MMP-2 [36]. Taken together, these observations suggest that the combination of VEGF-induced ECM breakdown and HA-mediated ECM augmentation may favorably condition the extracellular matrix for initiation of an angiogenic response.

Although the endothelial mitogenic capacity of VEGF is well established, vessels elicited by VEGF acting by itself are too porous for efficient perfusion. In contrast, although Ang-1 is not thought to be an effective promoter of endothelial mitosis itself, it is associated with the remodeling of forming microvessels to a higher-order structure, as well as with the recruitment of pericytes and smooth muscle cells to the vascular network [37]. Ang-1 acts through the Tie2 receptor and its pathway, and is thought to potentiate sprouting, survival, and antipermeability effects [38] surrounding neovessel maturation. Defects in Ang1/Tie2 signaling result in vascular dysmorphogenesis and substantial reduction of mural cell association with blood vessels [39]. Further, the addition of Ang-1 to mural cell-deficient capillary networks has been shown to restore a hierarchical vasculature, and has been found to ameliorate retinal hemorrhage and edema [40].

Although numerous biopolymer carrier systems for cytokine or drug delivery have been described, none of the other systems offer the combination of strength, stability, *in situ* biodegradation without systemic sequelae and ability to interact synergistically with growth factors shown by these HA-based gels [15]. As a result, even very low doses of delivered factors can produce a highly effective desired biologic response. Those properties offer the opportunity to design “intelligent” implants that participate in the tissue response, rather than supplying only an inert conduit for sequestered agents. One important application of this biomaterial is the regulated delivery of dual vascular growth factors to generate a controlled, localized angiogenic response. From a broader perspective, however, the ability to regulate tissue development by controlling the local availability of multiple cytokine combinations will provide a powerful approach to managing a broad number of pathologic processes. Therapeutic techniques may ultimately be greatly enhanced by the temporal and spatial regulation of signals achievable *in vivo* in this way.

5. CONCLUSIONS

The ability of dual growth factor-loaded, hyaluronic acid-based hydrogel films to stimulate an *in vivo* angiogenic response has been investigated in a mouse model, in both the presence and absence of 0.3% w/w modified heparin in the gels. By sequestering and providing localized *in vivo* release of the growth factors without loss of their biologic effectiveness, the presence of heparin in the gels allows the rate of release to be regulated and sustained over a period of weeks. Thus these hydrogels are capable of emulating the functions provided by the heparan sulfate proteoglycan content of native ECM. As evaluated by a neovascularization index, treatment with implants containing both VEGF and Ang-1 produced continuing increases in vascularization from day 7 to day 14 in the absence of heparin. In contrast, in its presence the vessel density declined with time. However, histopathologic evidence showed that animals receiving heparin-containing implants with both growth factors developed more mature microvessel networks than animals receiving implants either lacking heparin or containing only VEGF or only Ang-1. The resulting vessel networks showed defined borders and no evidence of inappropriate permeability. The ability to stimulate localized microvessel growth at controlled rates by administration of multiple growth factors via GAG carrier films will provide a powerful therapeutic tool.

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Figure 1.
Chemical structure of crosslinked HA-DTPH and Hp-DTPH

Figure 2a

Figure 2b

Figure 2c

Figure 2d **Figure 2.**

Hematoxylin and eosin stained representative images of ear tissue for different implant types, 400 ×, (a) contralateral ear, (b) HA+Ang-1+VEGF implanted ear, day 14 post-surgery, (c) HA/Hp+Ang-1+VEGF implanted ear day 7 post-surgery, (d) Ang-1+VEGF preloaded HA/Hp implant day 14 post-surgery. Ca—capillary, Ch—chondrocytes, E—erythrocytes, En—endothelial cell, H—hair follicle, L—polymorphonuclear leukocyte.

Figure 3a

Figure 3b

Figure 3. Microvessel density for non-heparinized HA implant treatment groups at (a) day 7 and (b) day 14 post-surgery. Results are shown for the control cases of surgery without implant placement, aqueous Ang-1, aqueous Ang-1+VEGF, HA+Ang-1, and the experimental case HA+Ang-1+VEGF; mean $\pm$ s.d., $n = 6$.

Figure 4a

Figure 4b **Figure 4.**

Microvessel density for heparin-containing HA implant treatment groups at (a) day 7 and (b) day 14 post-surgery. Results are shown for the control cases of surgery without implant placement, HA/Hp without a growth factor, HA/Hp+Ang-1, HA/Hp+VEGF, and the experimental case HA/Hp+Ang-1+VEGF; mean $\pm$ s.d., $n = 6$.

Figure 5a

Figure 5b **Figure 5.**

Neovascularization index for non-heparinized HA implant treatment groups at (a) day 7 and (b) day 14 post-surgery. Results are shown for aqueous Ang-1, aqueous Ang-1+VEGF, HA +Ang-1, and the experimental case HA+Ang-1-VEGF; mean $\pm$ s.d., $n = 6$. *NI* is defined in the text, Eq. (1).

Figure 6a

Figure 6b

Figure 6. Neovascularization index for heparin-containing HA implant treatment groups at (a) day 7 and (b) day 14 post-surgery. Results are shown for HA/Hp, HA/Hp+Ang-1, HA/Hp+VEGF, and the experimental case HA/Hp+Ang-1+VEGF; mean $\pm$ s.d., $n = 6$. *NI* is defined in the text, Eq (1).