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Collagen and collagen-glycosaminoglycan matrices as carriers for growth factors

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Abstract. Tissue engineering is an emerging interdisciplinary field that applies the principles of engineering to the life sciences, with the aim of developing biological substitutes that restore, maintain or improve tissue function. In this process, extracellular matrix, cells and regulatory signals are important in guiding, modulating and facilitating regenerative events.

Cellular activities are regulated by a large number of polypeptides which behave as growth modulating factors. Such growth factors can either stimulate or inhibit cell division, differentiation, migration or expression. The effects of such factors are cell type-dependent and can vary with the frequency and way of administration. As we increase our understanding of growth factor functions and their clinical applications, the need for useful pharmaceutical forms becomes more apparent. Growth factor targeting to responsive cells and maintenance of adequate tissue levels becomes essential, particularly in view of their sometimes opposite effects on various cells and the dose dependence of their response.

The extracellular matrix provides a scaffold for cell growth, differentiation and may help to eventually regenerate tissues. Since collagen is a major constituent of extracellular matrices and connective tissues, its use in designing a synthetic matrix becomes of special interest. When growth factors contain collagen-binding domains, they can be targeted to collagen matrices, their activities localized, and together with the collagen matrix, synergistically affect the biological activities of cells. Therefore, collagen matrices impregnated with growth factors become potentially useful for tissue repair and organ regeneration, stimulating cell growth and extracellular restoration as well as remodeling.

We have developed collagen-derived matrices and are in the process of investigating their interactions with cells and related growth factors for tissue regeneration and repair. In this addition, we will discuss means of modulating growth factor release including the use of recombinant protein strategies for targeting their delivery.

Keywords. Collagen; glycosaminoglycans; growth factors; fibronectin.

1. Introduction

1.1 The extracellular matrix

Collagen is the single most abundant animal protein in mammals, accounting for upto 30% of all proteins. The collagen molecules, after being secreted by the cells, assemble into characteristic fibers responsible for the functional integrity of tissues such as bone, cartilage, skin, and tendon. They provide a structural framework for other tissues, such as

blood vessels and most organs. Cross-links between adjacent molecules are a prerequisite for the collagen fibers to withstand the physical stresses to which they are exposed.

To understand the physical properties of connective tissues such as cartilage and intervertebral disks, it is important to have an understanding of the salient features of the proteoglycan molecules. There are essentially two types of glycosaminoglycans: those with weak negative charges (e.g., hyaluronic acid) and those with strong negative charges (e.g., the chondroitin sulfates, heparins, and dermatan sulfate, the latter comprising the largest bulk of the proteoglycans). Their distribution and physiochemical characteristics, which contribute to distinct functions, are also unique. Hyaluronic acid, with weak negative charges associated with the carboxylic acid residues present in glucuronic acid, has a tendency to form hydrated gels and can therefore contribute significantly to the viscoelastic fluidity of synovial fluid and to the turgency of the skin of an infant.

On the other hand, the negatively charged polysaccharides that contain sulfonic acid residues are able to develop strong ionic bonds with the positively charged amino acids, particularly lysine, hydroxylysine, and arginine on the surface of the collagen fibers. Such tissues are more compact, resilient, and less hydrated and exhibit the viscoelastic behavior typified by hyaline articular cartilage. They are also more collagenous than their fluid counterparts. Synovial fluid has no collagen, the vitreous body of the eye has only small amounts of type II collagen, and the skin of a newborn rabbit has less than 2% collagen; this in contrast to a three-month-old rabbit, which has more than 15% collagen. Fetal skin has so little collagen that its wounds can heal without generating scars.

The physiochemical properties of the various types of connective tissues, their viscoelastic properties, the diffusion of macromolecules and of small ions through their midst, and the exclusion of molecules of various molecular masses (e.g., immunoglobulins) are, understandably, different [1].

2. Growth factors

Growth factors are part of a large group of polypeptides that transmit signals affecting cellular activities. Cells may communicate with each other through direct molecular interactions involving their cell membranes, as a result of the movement of molecules across gap junctions, or less directly by virtue of molecules, such as peptides or steroids, that can act locally or systematically to modulate cell functions. Growth factors are included in the latter group.

The word "growth factors" is probably not ideally suited to describe the function of these polypeptides because they are not always promoting growth but in many instances may hinder it or even direct cells to produce a variety of products for their own consumption or for export. In essence, they act as modulators of cellular activities. Polypeptide hormones, although performing similar functions mediated by related pathways, are mostly considered as a separate group of cell growth-modulating molecules. The term cytokine, on the other hand, although often used synonymously with growth factor, is generally reserved to describe factors associated with cells involved in immune surveillance.

Growth factors can either stimulate or inhibit cell division, differentiation migration or gene expression depending on the cells involved [2]. Depending on the concentration present in the cellular environment, growth factors can act in an opposing manner and up- or down-regulate the synthesis of receptors. In general, both growth factors and m-RNAs that code for them turn over very rapidly. They usually exist as inactive or partially active

precursors that require proteolytic activation, and may need to bind to matrix molecules for activity or stabilization. They may perform different functions on different cell types: for instance TGF-β is stimulatory for fibroblasts and inhibitory for keratinocytes.

Depending on the proximity of their sites of synthesis to sites of action, growth factors have been classified as endocrine, paracrine, or autocrine[2], reflecting a progressive decline in distance. For short range interactions the terms juxtacrine and intracrine have sometimes been used[3–5]. Hundreds of growth factors have been described so far, some much better characterized than others. Taking into account structural homologies they have been grouped into at least 20 families and superfamilies[6–8].

In contrast to the early work on hormones, which are isolated from glandular tissues and have a particular target organ, growth factors have been isolated from whole organisms (embryos), organs, tissue or cell cultures. Conditioned media from cultured cells have proven to be a useful source of such factors.

With the increased availability of growth factors derived from cultured human cells and expanded through recombinant technologies, coupled with an increasing understanding of their functions and clinical applications, the need for useful pharmaceutical forms is becoming more and more apparent. Growth factor targeting to responsive cells and maintaining adequate pharmacological levels becomes essential, particularly in view of their different effects on various cells and the dose dependence of their response.

An important issue is the short biological half life of growth factors. For example PDGF, an important growth factor first isolated from platelets, cannot be detected in the circulation, and when injected intravenously its half-life is less than 2 minutes[9]. It is present in many cells, in addition to platelets, and has been implicated in the pathogenesis of diseases such as atherosclerosis, bone marrow and lung fibrosis, neoplasia and chronic inflammation. Similarly TGF-β, which because of its intrinsic binding characteristics circulates in the blood stream in a latent form with a half-life of 90 min, while the active form of TGF-β1 would be cleared from the circulation in a matter of a few minutes[10].

3. Effects of immobilized growth factors on cellular activities

Only recently has interest on the effects that immobilized growth factors have on cells begun to emerge. Insulin immobilized on surface-hydrolyzed poly(methyl methacrylate) membranes caused a growth acceleration of mouse fibroblasts[11]. For immobilization, surface-hydrolyzed PMMA membranes were immersed in phosphate-buffered saline containing a water-soluble carbodiimide. Insulin of various concentrations was added and the coupling reaction continued for 1 h at 30°C. To introduce a spacer arm between the insulin and the PMMA membrane, the surface-hydrolyzed PMMA membrane was coupled with α,ω-diaminopolyoxyethylene (M_r 3000) and insulin connected to the amino terminal. It was found that insulin remained immobilized on the surface of these nonbiodegradable membranes and interacted specifically with receptors existing on the fibroblast. The growth acceleration by immobilized insulin was enhanced by introduction of this spacer arm. The amount of receptors present on the membrane of fibroblasts, after culturing with insulin immobilized on nonbiodegradable polymer surfaces, was much higher than that after culturing with free insulin, implying suppression of down-regulation by immobilized insulin. Coimmobilization of insulin and fibronectin was also very effective in accelerating cell growth[12]. When insulin, transferrin and collagen were immobilized on the surface of hydrolysed poly(methyl methacrylate) films the growth factors remained immobilized without detachment and accelerated cell growth in a more

potent manner than free or adsorbed growth factors. Immobilized collagen enhanced the flattening of adhered cells in the early stages of cell adhesion, but did not enhance cell growth significantly [13].

Cell guidance on artificial surface is another area which will surely continue to develop in connection with the design of drug delivery systems. Precise regional control of cell adhesion, migration, and growth on artificial substrates could be used to create two-dimensional tissue structures. These fundamental cellular events are mediated by adhesive proteins, such as fibronectin, vitronectin, and collagen, adsorbed from serum or preadsorbed on artificial substrates. Non-adhesive surfaces usually are produced by incorporation of highly swollen non-ionic layers onto artificial surfaces, which prevents adsorption of any protein, including adhesive proteins. Surface modification has successfully produced hydrophilic and non-hydrophilic regions when photomasks were placed on coated surfaces and subsequently irradiated with ultraviolet light [14–15]. This approach based on differential cell adhesion, enables to control cellular potentials such as adhesion, migration, and axonal growth and may provide a research tool for the building of functional artificial neural networks with given patterns, particularly if coupled with growth factors such as NGF.

4. Construction and characterization of a fusion protein with EGF and the cell-binding domain of fibronectin

In addition to approaches that rely on the adsorption of ionically bound, physically entrapped or covalently bound factors to resorbable matrices, some novel approaches rely on the construction of fusion proteins which include a growth factor and cell or matrix binding domains.

Fibronectins (FN) are multifunctional cell-adhesive glycoproteins present in plasma and the extracellular matrix. FN contains several functional domains, including a cell-binding domain, which contributes to the cell-adhesive function of FN. The tetrapeptide Arg–Gly–Asp–Ser (RGDS) has been identified as a recognition signal for cell adhesion [16].

With this in mind an expression system was constructed for C-EGF, a fusion protein made of a fragment of the cell-binding domain of human fibronectin (FN) bound with epidermal growth factor (EGF). C-EGF was produced in *Escherichia coli* HB101 cells carrying the recombinant plasmid pCE102 as inclusion bodies, which were solubilized and refolded after purification. The C-EGF had both cell-adhesive and EGF activities [17] (figure 1).

The plasmid used, pCE102 coded for a fusion protein of the cell-binding domain of human FN (Pro^{1239}–Ser^{1515}), with 277 amino acids, bound via Met–Ala to human EGF. *E. coli* HB101 cells carrying the plasmid pCE102 produced a 35-kDa protein, which was detected by SDS-PAGE of the whole-cell lysate and by immunoblotting with use of monoclonal antibodies. The 35-kDa protein, named C-EGF, accounted for about 20% of the total cell protein by densitometric analysis and was produced as inclusion bodies. The inclusion bodies were washed extensively, solubilized with a buffer that contained 6 M urea, and then purified by ion-exchange chromatography.

The purified C-EGF was refolded in glutathine/oxidized glutathine (10:1 ratio) and had the same amount of cell-adhesive activity as an unfused fragment of the cell-binding domain, C-279 (Pro^{1239}–Met^{1517}), with 279 amino acids. The EGF activity of C-EGF was also compared with that of recombinant human EGF. C-EGF was almost the same as human EGF in its stimulation of [^{3}H]-thymidine uptake.

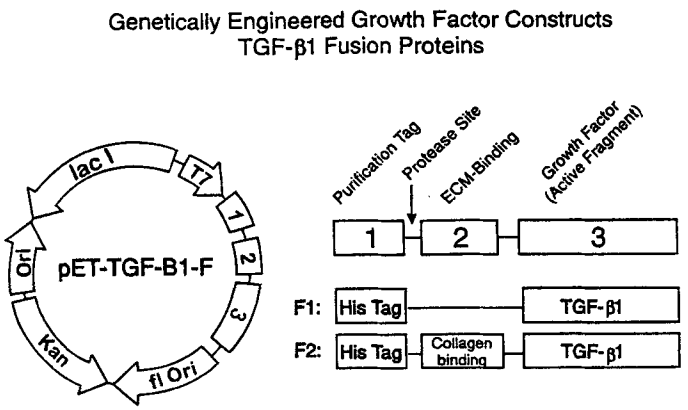

Figure 1. A schematic representation of the genetically engineered TGF-β1, (TGF-β1-F1 and TGF-β1-F2) fusion protein constructs. The TGF-β1-F2 construct contains the collagen binding decapeptide sequence (WREPSFMALS) while the TGF-β1-F1 construct does not contain the collagen binding sequence.

5. Recombinant TGF-β with affinity to ECM components as a bioactive wound healing enhancer

It has become apparent that for TGF-β to be effective as a therapeutic agent with minimum side effects, the targeting of TGF-β to specific sites, the controlled release of the active molecule, local containment of the active molecules, and long-term protection of the active growth factor must be taken into consideration. For this reason we have recently genetically engineered TGF-β1 fusion proteins which contain, in addition to the active human TGF-β protein fragment, a purification tag and different binding domains which have the affinity for extracellular matrix components [18–20]. The purification tag comprises of a hexapeptide of $(His)_6$ which binds tightly to a Ni-NTA column and the binding can be dissociated with acidic buffer or imidazole (figures 2 and 3). The matrix component binding sequence is either a modification of a decapeptide sequence (WREPSFCALS) within the von-Willebrand factor [21] or a fibronectin-binding peptide sequence (Gly–Gly–Trp–Ser–His–Trp) derived from thrombospondin [22]. The purification tag and the matrix binding domain are linked to the active TGF-β domain through (Gly–Gly) linkers.

The three initial fusion proteins TGF-β1-F1, TGF-β1-F2 and TGF-β1-F3 were obtained by designing the DNA constructs which were then transfected into *E. coli* induced expression in high yield. The expressed proteins, as insoluble inclusion bodies from *E. coli*, were solubilised in urea and renatured in a redox-coupled refolding buffer system to yield the active TGF-β fusion proteins. TGF-β1-F1 contains only the active TGF-β1 fragment and the purification tag. TGF-β1-F2 has the additional collagen binding domain and TGF-β1-F3 has the fibronectin binding domain instead. Our results showed that the purification tag was effective in providing a quick one-step purification process to obtain virtually pure TGF-β1 (225). All three recombinant fusion proteins are biologically active judged by the *in vitro* mink lung epithelial (Mv1Lu) cell inhibition assay and the stimulation assay of mouse NIH 3T3 fibroblast proliferation. We have also demonstrated tight binding of the TFG-β1-F2 to collagen and gelatin. The TGF-β1-F1 fusion protein was incorporated into a collagenous matrix and implanted as a bone

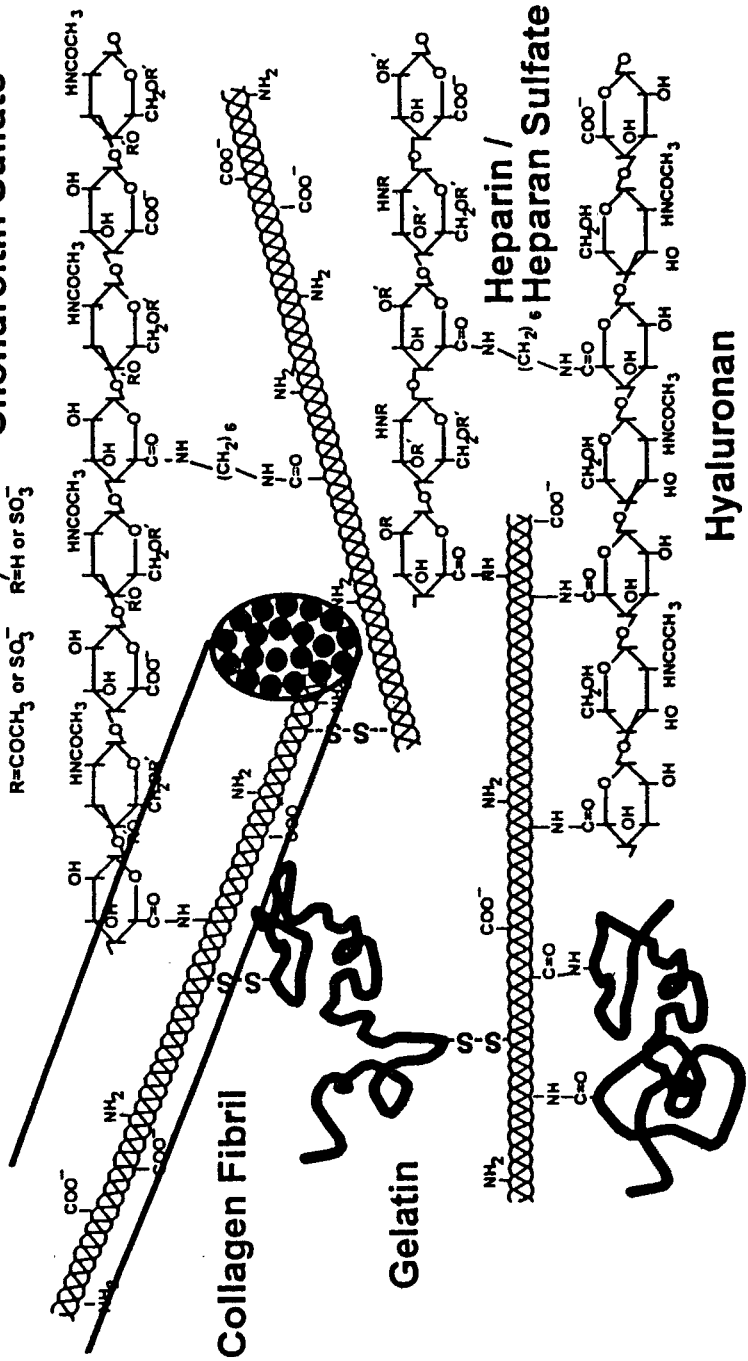

Figure 2. Modified, reconstituted or engineered biosynthetic extracellular matrices containing various proportions of native components, rearranged and crosslinked to each other may provide suitable carriers for the local delivery of such factors (Huang Lee and Nimni).

substitute in large cranial defects in rats. Histology of the explants showed that the defects were filled with newly formed bone in contrast to the collagen controls which did not stimulate bone formation and which developed a marked inflammatory response and adhesions.

The above studies demonstrate the potential of targeted growth factors. They also contribute to the elucidation of the role of TGF-β during the inflammatory stage and to its osteoinductive and would repair potential. In some instances, as the ones described, adsorption of the growth factor on a carrier matrix can prove to be of added value, particularly if the matrix used, as in the case of purified telopeptide-free fibrous collagen, can serve as a scaffolding to promote tissue or organ regeneration [23] (figure 2).

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