Biotinylation of implantable collagen for drug delivery

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INTRODUCTION

Delivery of biologically active compounds has been studied experimentally, and practiced clinically with a wide variety of physical and chemical implants.[1,2] Implantable collagen has been used for cosmetic surgery in an injectable form,[3] for dermal wound repair,[4] for internal surgical hemostats,[5] and for experimental tissue and organ substitutes.[6–8] Particularly, this laboratory has combined a porous and resorbable collagen-based substrate[8] with cultured human keratinocytes[9] and fibroblasts to prepare a skin substitute[10] that is effective for closure of noninfected, excised burns in humans.[11]

The present report describes application of the biotin-avidin biochemistry[12] to attach biologically active molecules to implantable collagen. Bovine skin collagen is modified by covalent addition of biotin (Vitamin H) from biotinyl-*N*-hydroxy-succinimide (BNHS). An *in vitro* assay is adapted in which horseradish peroxidase (HRP) is used in a chromogenic reaction to identify biotinylated collagen on nitrocellulose paper.[8] Binding of avidinylated-HRP to biotinylated collagen, or of biotinylated-HRP to biotinylated collagen plus avidin, is specific and concentration-dependent to the biotinylated collagen ligand. Attachment of Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) to implantable collagen has also been reported.[13] Results of the present study indicated that any combination of compounds to which biotin can be added, may also be attached to implantable collagen for drug delivery.

EXPERIMENTAL PROCEDURES AND RESULTS

Procedures

Covalent addition of biotin to primary amino groups of polypeptides occurs by the condensation reaction shown in Figure 1. Biotinyl-*N*-hydroxy-succini-

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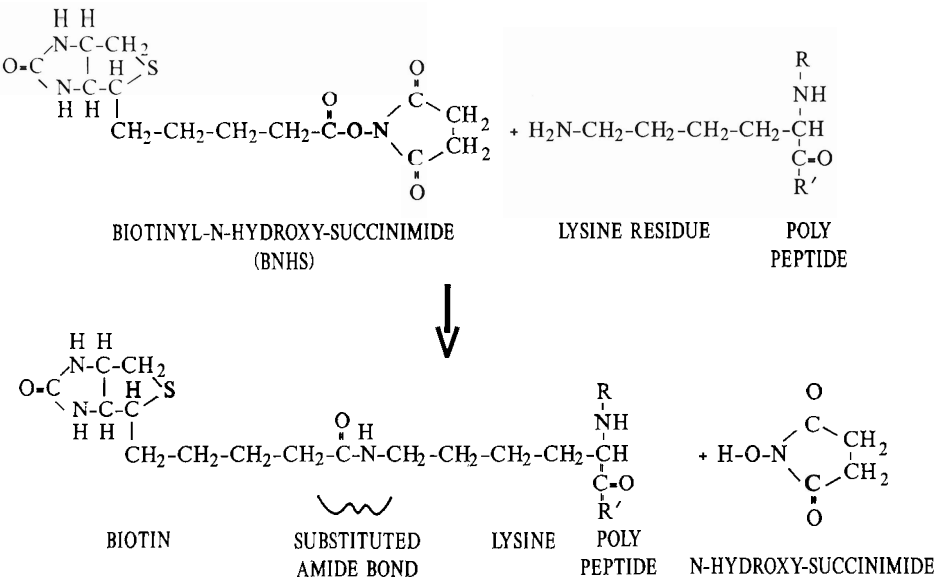

Figure 1. Chemistry of biotinylation of polypeptides. Biotinyl-*N*-hydroxy-succinimide (BNHS) reacts with ε-amino groups of lysine residues to form biotinyllysine by generation of a substituted amide bond. *N*-hydroxy-succinimide is released from the reaction.

mide (BNHS) reacts with primary amino groups and adds biotin by a substituted amide bond to ε-amino groups of lysine residues of proteins. After completion of the reaction, *N*-hydroxy-succinimide is released and removed from the biotinylated compound by dialysis.

Spot test assay

The spot test procedure described by Boorsma,[14] was modified in this study. Figure 2 shows diagrammatically the assay formats for the Labeled Avidin to Biotin (LAB) procedure [Fig. 2(A)], and for the Bridged Avidin to Biotin (BRAB) procedure [Fig. 2(B)]. Collagen (5 mg/mL) conjugated with biotin or nonbiotinylated collagen was diluted 1:2, 1:3, 1:5, 1:10, and 1:30 using 0.05*M* Tris buffer, pH 7.6. Three microliters of each dilution was spotted onto the NC paper, allowed to air dry, the NC paper was soaked until fully hydrated, and then rinsed twice with 0.05*M* Tris plus 0.05% Tween 20, and drained. For the Labeled Avidin to Biotin (LAB) assay, 5 μL of 100 μg/mL avidin-HRP were applied over each spot and allowed to incubate for 30 min. For the Bridged Avidin to Biotin (BRAB) assay, 5 μL of 100 μg/mL free avidin were applied to each spot and allowed to incubate for 30 min. The NC paper was rinsed 3 $\times$ 10 min with Tris-Tween buffer and drained. Next, 5 μL of biotin-HRP was applied to each spot and incubated for 30 min. For both the LAB and BRAB assays, the NC paper was rinsed 3 $\times$ 10 min with the Tris-Tween buffer and drained. Then, 5 μL of a chromogen solution containing 0.5 mg/mL diaminobenzidine in 0.05*M* Tris, pH 7.6, containing 0.01% H_2O_2,[15]

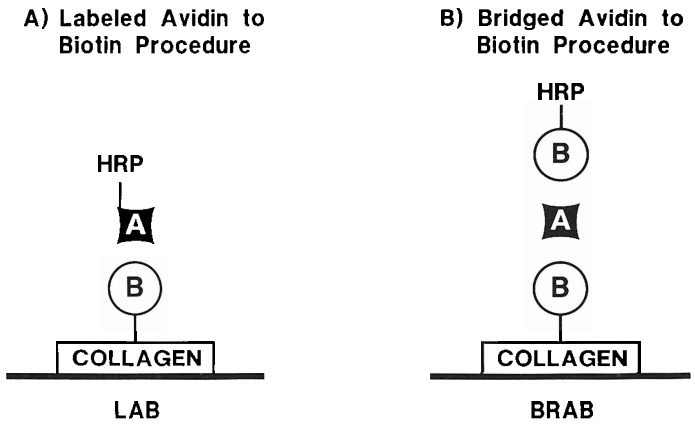

Figure 2. Schematic representation of biotin-avidin binding system. (A) Labeled Avidin to Biotin (LAB) procedure. HRP is covalently bound to avidin. (B) Bridged Avidin to Biotin (BRAB) procedure. Free avidin is used as a bridge between two biotinylated compounds, biotinylated collagen and biotinylated HRP.

was applied to each spot, developed for about 3 min, and quenched with the Tris-Tween buffer.

Specificity of the LAB reaction was tested by: (a) preincubation of avidin-HRP with free biotin, (b) pre-incubation of biotinylated collagen with free avidin, or (c) substitution of nonbiotinylated collagen for biotinylated collagen. Specificity of the BRAB reaction was tested by: (a) removal of avidin from the reaction, (b) substitution of nonbiotinylated HRP for biotinylated HRP, (c) substitution of nonbiotinylated collagen for biotinylated collagen, and (d) preincubation of the biotinylated collagen-avidin complex with free biotin (as BNHS) before exposure to biotinylated HRP.

Quantitative digital matrix photometry

A photometry instrument based upon a design by Neeley[16,17] was used to measure the reflectance, and conversely, the amount of light absorbed (540 nm) by each colored spot on the NC paper.

Results

Dose-dependent and reaction-specific binding of avidin-HRP to biotinylated collagen in the LAB assay is shown in Figure 3. Quantitative results of the LAB and BRAB assays are shown in Table I. All tests demonstrated statistically significant differences (Students t test, $p < 0.05$) from complete reactions of 5 mg/mL biotinylated collagen with avidin-HRP in the LAB assay, or with avidin plus biotin-HRP in the BRAB assay.

DISCUSSION

The present studies demonstrate a principle that biotin-avidin binding may be used for association of collagen and peroxidase molecules that have no in-

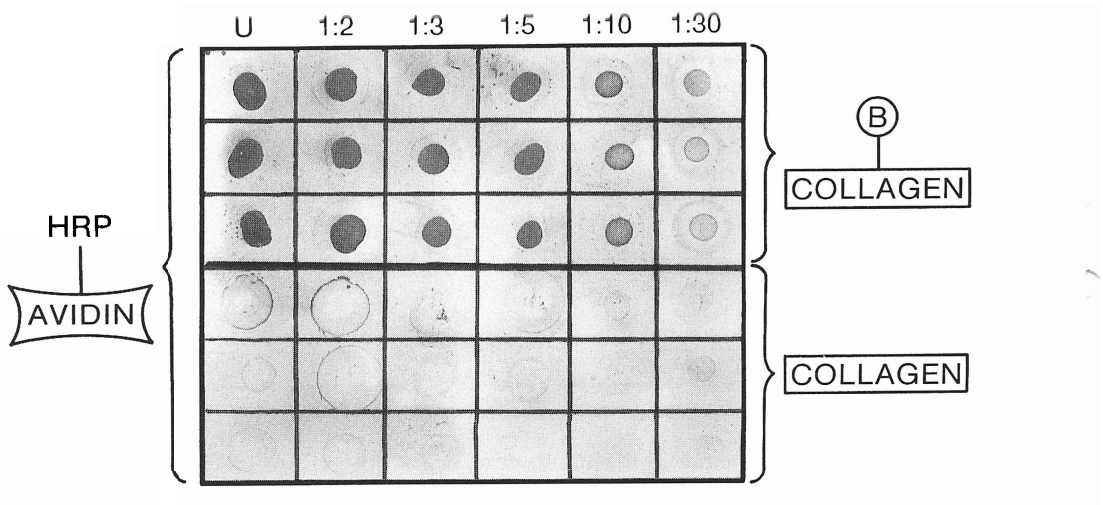

Figure 3. Specific and concentration dependent binding of horseradish peroxidase to biotinylated collagen in the Labeled Avidin to Biotin (LAB) procedure.

herent affinity. Furthermore, these findings support the hypothesis that any combination of biotinylated compounds may be bound to implantable collagen in any molar ratio. Specific binding of peptide growth factors, EGF and bFGF, to collagen using the BRAB procedure has been reported.[13] They also retained their respective mitogenic activities on cultured epidermal keratinocytes after biotinylation, in the absence of biotinylated collagen. Those findings support the hypothesis that biotinylated compounds can be used to modulate cellular responses in wound treatment. Other studies from these investigators have described the combination of implantable collagen and cultured cells[8–11] to form skin substitutes that have been demonstrated to close burn wounds. Extension of the present studies to use biotinylated collagen as the substrate for cultured cells for transplantation provides a model for new approaches to modulation of cells and tissues in the wound. Particularly, implantable collagen of tissue substitutes may act as a mechanical vehicle for transportation of cultured cells in transplantation, and as a biochemical vehicle for delivery of pharmacologically active drugs.

Several important questions were not addressed in these studies. It has not yet been shown whether the activities of biotinylated compounds are retained if bound to biotinylated collagen. Neither the mechanism nor rate of release of compounds from biotinylated collagen are known presently. However, the rate at which drugs are released is expected to be no less than the rate of degradation of the implanted collagen. Immunogenicity of biotinylated collagen, of free avidin, or of the BRAB complex has not been studied, and may influence the application of this technique. It may be noted, however, that biotinyllysine is a naturally occurring complex that acts as a carrier of carbon dioxide during the action of certain carboxylating enzymes (e.g., acetyl-CoA carboxylase and propionyl-CoA carboxylase).[18] With further studies to address these important questions, application of principles established

TABLE 1
Quantitative Assays for Percentage Maximum Binding* of Peroxidase (HRP) to Biotinylated Collagen

		LAB Assay				BRAB Assay			
Reacted sequentially with		Av-HRP	Av 100 Av-HRP	Av 1000 Av-HRP	Biotin+ Av-HRP	Av B-HRP	B-HRP	Av HRP	Av Biotin B-HRP
Biotinylated collagen									
Dilution	1:1	94.2 ± 2.0	41.5 ± 3.7	17.2 ± 1.3	38.9 ± 1.1	88.9 ± 3.6	15.8 ± 3.7	4.5 ± 1.9	3.4 ± 1.0
	1:3	57.4 ± 4.5				46.4 ± 0.4			
	1:10	24.8 ± 2.3				20.9 ± 3.0			
	1:30	9.0 ± 1.6				7.0 ± 1.7			
Nonbiotinylated collagen									
Dilution	1:1	3.6 ± 0.8				18.9 ± 4.3			
	1:3	3.0 ± 0.7				9.1 ± 1.9			
	1:10	2.1 ± 0.4				5.6 ± 1.1			
	1:30	1.3 ± 0.4				6.7 ± 1.9			

Data collection and statistical analysis: Quantitative data were collected from duplicate experiments performed in triplicate to give a minimum of six measurements per condition. Differences between pairs of conditions was considered statistically significant if Student's t-test produced a value of $p < 0.05$.

*Data are expressed as % maximum binding (mean ± SEM), with maximum binding defined as the absorbance produced by staining of 5 μL of 100 μg/mL avidin-HRP (for LAB assay) or 5 μL of 100 μg/mL biotin-HRP (for BRAB assay) applied directly to nitrocellulose paper, reacted as above.

in this study may provide an alternative mechanism for drug delivery with implantable collagen.

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