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# Gastrointestinal Absorption of Insulin-Like Growth Factor in the Mouse in the Absence of Salivary Insulin-Like Growth Factor Binding Protein

*Yoichi Nakagawa,* Gregory E. Oxford,† Katsunori Ishibashi,* Hideo Yamamoto,*† Nobuko Maeda,‡ Elizabeth Bowen,† Jason Brayer†§ and Michael G. Humphreys-Beher†‖¶*

Departments of *Oral and Maxillofacial Surgery, and ‡Bacteriology, Tsurumi University Dental School, Yokohama, Japan; and Departments of †Oral Biology, §Pathology, and ‖Pharmacology and Therapeutics, University of Florida, Gainesville, FL, U.S.A.

**ABSTRACT.** Based on previous observations of the presence of both insulin-like growth factors I and II (IGF-I and IGF-II) in murine saliva (Kerr *et al.*, *Biochem Pharmacol* **49:** 1521–1531, 1995), the saliva from BALB/c and Non-obese diabetic (NOD) mice was examined for the presence of insulin-like growth factor binding proteins (IGFBPs). Using a western-blot type ligand binding assay with $^{125}$I-labeled IGF-I, a series of binding proteins with molecular masses ($M_r$) between 25 and 45 kDa were detected in the sera, but not saliva, from both BALB/c and diabetic NOD mice. In the diabetic NOD mice, there were detectable changes in the concentrations of several of the IGFBPs relative to BALB/c mice. Using specific antibody to the binding proteins, one of these was identified as IGFBP-2. Gavage administration of [$^{125}$I]IGF-I indicated substantial uptake from the gastrointestinal tract and significant tissue distribution. There was an increase in serum concentrations of radiolabeled IGF-I in diabetic NOD mice over that in BALB/c mice but less recovered from most of the tissues. Intact $^{125}$I-labeled IGF-I was extracted and purified from various tissues, following gavage, and shown to retain biological activity. Thus, the uptake of biologically active IGFs from saliva would appear to take place independently of specific binding proteins. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:233–240, 1997.

**KEY WORDS.** growth factors; oral cavity; wound healing; secretion

The protein constituents of saliva impart a number of biological functions that are intimately involved in the maintenance of oral health. These various roles include lubrication of soft and hard tissue of the oral cavity, remineralization of the enamel, initial digestion of ingested food stuffs, antimicrobial activity for the control of infectious agents, and maintenance of mucosal integrity [1–3]. Proteins that have been implicated in the maintenance of mucosal integrity and oral wound healing specificity include growth factors present in saliva [2, 3]. Other salivary proteins involved in this process include those that modulate bacterial growth (lysozyme, salivary peroxidase, histatins), as well as mucous glycoproteins (mucin, proline-rich proteins) that protect the oral soft tissues from desiccation [1, 2]. Since the identification of EGF** in the submandibular gland of mice [4, 5], the salivary glands have been reported to synthesize and secrete nerve growth factor [6–8], transforming growth factor-α and -β [9–11], insulin [12–14], IGF-I and -II [15, 16], and fibroblast growth factor [17].

While a major portion of the body's growth factor is derived from saliva, the biological significance for these proteins remains unresolved. It is well-recognized that repair of oral soft tissue is more rapid than repair of cutaneous wounds. EGF is by far the most understood growth factor for its potential to modulate wound healing rates. EGF, synthesized by the salivary glands and secreted into saliva, appears to have a systemic effect on skin and gastric wound healing processes *in vivo* [18–20]. Removal of the submandibular gland leads to an increase in intestinal ulceration [21, 22]. Oral wound healing, removal of molars, and orthodontic tooth movement cause an increase in saliva-derived EGF or modulation of these events *in vivo*, respectively [23–26].

¶ Corresponding author: Michael G. Humphreys-Beher, Ph.D., Department of Oral Biology, P.O. Box 100424, University of Florida, Gainesville, FL 32610. Tel. (352) 846-0797; FAX (352) 392-3070; E-mail: h-beher@dental.ufl.edu

** *Abbreviations:* EGF, epidermal growth factor; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; NOD, Non-obese diabetic; MEM, minimal essential medium; FBS, fetal bovine serum; IDDM, insulin-dependent diabetes mellitus; TCA, trichloroacetic acid; PVDF, polyvinyldifluoride nitrocellulose; and TBS, Tris-buffered saline.

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Insulin and the IGFs are homologous growth hormones found in serum and generate similar biological responses in a wide variety of cell types [27–30]. The biological activity of IGF-I and -II is mediated by activation of receptor tyrosine kinase activity and the phosphotyrosine second messenger signal transduction cascade [30]. IGFs function in an autocrine/paracrine manner to regulate proliferation and differentiation of bone cells [31]. Furthermore, the activity of IGFs appears to be regulated by the presence of a specific set of serum proteins termed IGFBPs (IGFBP-1 through 6; [31, 32]). Various organs such as the pituitary, renal glands, and pancreas regulate the levels of circulating growth factor by influencing the level of IGFBPs in serum [32].

The ability of saliva-derived growth factors to maintain an ability to influence organ homeostasis and wound healing rates is dependent on their ability to cross the stomach mucosal barrier and enter the circulation while retaining its functional potential. Studies with EGF have indicated both a gastrointestinal and sublingual uptake and dissemination of biologically active growth factor to various tissues [33–35]. This suggests that saliva-derived growth factors retain activity and contain the capacity to elicit a biological response at sites distal to their synthesis [35]. The presence of IGF-I and IGF-II in saliva led us to investigate whether these growth factors are bound to specific binding proteins in saliva. Although we did not detect IGFBPs in saliva, it was determined that IGF-I could be distributed systemically by passage through the gastrointestinal mucosa in a form that retained biological activity.

## MATERIALS AND METHODS

### *Materials*

Antibodies to IGFBP-1, -2, -4 and -5 along with the IGFBP-1 antigen were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Recombinant human IGF-I and $^{125}$I-labeled growth factor were obtained from Amersham (Arlington Heights, IL). BALB/c and NOD mice (25–35 g) were obtained from the Department of Pathology, University of Florida. Typically, BALB/c and pre-diabetic NOD mice had fasting blood glucose levels of 180–240 mg/dL, while diabetic mice had levels of 280 mg/dL or higher as determined using Boehringer Mannheim (Indianapolis, IN) Chemstrip IG reagent strips [14]. Treatment of mice, including insulin supplementation, was in accordance with guidelines set forth by the Institutional Animal Care and Use Committee (Approval 2112) at the University of Florida. All other reagents were of ultrapure quality and obtained from commercial sources.

### *Saliva and Serum Collection and Gland Preparation*

Whole saliva from male and female mice was collected by the method of Hu *et al.* [36]. Briefly, animals were given an intraperitoneal injection of pilocarpine (0.05 mg/100 g body wt) and isoproterenol (0.2/100 g body wt) dissolved in saline after which saliva was collected over a 10-min period by a hand-held micropipette. Animals were fasted for 18 hr before saliva collection. Whole blood was collected through cardiac puncture and clotted for 30 min at 4°. Serum was separated and removed from blood cells following centrifugation at 500 *g* for 5 min.

For the analysis of IGF uptake and tissue distribution, the animals were first mildly anesthetized with pentobarbital. Body temperature of anesthetized animals was maintained between 36.5 and 38° with an overhead lamp. Thirty minutes prior to killing the mice, radiolabeled growth factor ([$^{125}$I])IGF-I at $5 \times 10^5$ cpm in 100 μL saline) was supplied into the gut through gastrointestinal tract gavage using a syringe coupled with a Teflon tube for passive entry into the animal [35]. All tissues were identified by gross morphology, prior to their removal. The samples were washed in saline, weighed on a Mettler analytical balance, and placed on ice before determination of total tissue radiolabel content using a Beckman gamma counter [35].

### *Chromatographic Analysis of [$^{125}$I]IGF-I from Tissues*

After radioisotope quantitation of the tissue distribution of IGF-I, the growth factor was isolated for further characterization. Solubilization of radiolabeled growth factor in 1.5 mL of 1.0 N acetic acid was accomplished by incubation of the tissue slurries at 4° for 30 min. Insoluble denatured proteins were removed by centrifugation at 4° for 15 min at 10,000 *g* in a Sorvall RC-3B. The supernatant was removed and counted along with the pellet. The supernatant material was lyophilized to remove acetic acid, resuspended in 500 mL of PBS, and fractionated over a G-25 molecular sieve column (1.5 × 20 cm) using PBS as the chromatography buffer. The column eluate was collected in 1.0-mL fractions. The original [$^{125}$I]IGF-I and [$^{125}$I]EGF were used to calibrate the column for the elution profile of intact growth factor and separation from endogenous EGF [35].

### *Assay for Retained Biological Activity*

Radiolabeled IGF-I purified by column chromatography was examined for the ability to stimulate the proliferation of *in vitro* cultured fibroblasts. Human fibroblasts (courtesy of Dr. Thomas Hassell, Department of Periodontology, University of Florida) were cultured to confluency in Eagle's MEM containing 10% FBS. The cells were then plated into plastic 24 wells at $10^4$ cells/well and cultured for 24 hr in the same medium. Subsequently, the cells were washed twice with Hank's buffer and cultured for 20 hr with MEM without serum supplementation or MEM + 10% FBS, MEM + 1, 10, or 20 ng IGF-I, or MEM + 100, 200, or 300 μL of tissue extracted and purified IGF-I. At 20 hr, 0.05 μCi [$^3$H]thymidine was added to each well for an additional 4 hr. Each well was washed free of radiolabeled medium with Hank's solution after 4 hr of 0.05 μCi [$^3$H]thymidine incorporation. Fibroblasts were recovered by addition of 2 M urea for 15 min at 37°. Cells were lysed with the addition of 0.5 mL of PBS and 1 mL of 10% TCA to 100 μL of

sample. $^{3}$H-Labeled thymidine incorporation into DNA was assessed by TCA precipitation on glass fiber filters and counted in a Beckman LS 3000 liquid scintillation counter. All assays were performed in triplicate on three separate occasions.

### *Polyacrylamide Gel Electrophoresis and Western Blotting*

As described previously, 10 μL of whole saliva or 15 μL serum from BALB/c or diabetic NOD mice was separated on a 10% SDS-polyacrylamide gel [36]. Samples were mixed with sample buffer containing 0.5% SDS and 0.1% 2-mercaptoethanol, heated to 100° for 5 min, and applied to the gel. After separation by size, proteins were transferred to PVDF (Millipore, Bedford, MA) for 2 hr at 70 V. The protein blots were blocked in a solution of TBS containing 3% non-fat dry milk and 3% BSA and incubated at 23° in the same buffer containing a 1:500 dilution of primary antibody to IGFBP-1, -2, -4 or -5 for 12 hr. The blots were washed three times, incubated for 2 hr with a goat anti-rabbit alkaline phosphatase-conjugated second antibody, washed, and incubated with chromogenic reagents.

For ligand binding of [$^{125}$I]IGF-I to IGFBPs separated on PVDF membranes, saliva (20 μg), serum (10 μg), and salivary gland lysates (75 μg) were heated to 100° for 5 min in 0.2% SDS-containing sample buffer without 2-mercaptoethanol. Following electrophoresis and transfer to PDVF membranes, the filters were blocked with TBS containing 3% Tween 20 for 30 min followed by incubation in TBS containing 1% BSA and a 10-min wash in TBS containing 0.1% Tween 20 and 1% BSA [37]. Final incubation at 4° for 24 hr was carried out in TBS containing 0.1% Tween 20, 1% BSA and $5 \times 10^4$ cpm of [$^{125}$I]IGF-I. The filters were washed in two changes of TBS containing 0.1% Tween 20 for 15 min followed by three washes in TBS for 15 min, dried, and exposed for 72 hr at −80° to Kodak XAR-5 film.

A mobility shift analysis of IGF interaction with putative IGFBPs was performed in non-denaturing gels by incubating 10 μg of saliva or serum with $2.5 \times 10^4$ cpm of [$^{125}$I]IGF-I in a total of 30 μL TBS for 30 min at 4°. Ten microliters of a 4x gel sample buffer containing 0.2% Triton X-100 was added to the samples and applied to 10% polyacrylamide gels cast without SDS. Electrophoresis tank buffer consisted of Tris-glycine with 0.1% Triton X-100 [34]. Following electrophoretic separation, the polyacrylamide gels were fixed and dried, and exposed to X-ray film, as above, for 24 hr.

### *Statistical Analysis*

All values for [$^{125}$I]IGF-I distribution represent the mean cpm/g wet weight of tissue ± SEM for 4 experimental animals. For blood and saliva collection of IGFBPs, the polyacrylamide gels represent the typical profiles of 5 individual BALB/c or NOD samples. Tissue homogenates represent the pooling of glands from 3 animals. Each assay was performed on two separate occasions from three preparations of glands. All gels were analyzed by densitometry, and relative band intensities were analyzed by the Bonferroni paired comparisons test to determine significant difference between the means after a significant analysis of variance test. $P < 0.05$ was considered significant.

## RESULTS

Whole saliva and serum, as a control, were separated on polyacrylamide gels and probed with specific antibody to several IGFBPs to establish their presence in mouse saliva. As shown in Fig. 1, antibody to human IGFBP-2 consistently cross-reacted with a protein in the serum from BALB/c and diabetic NOD animals out of four antibodies tested (IGFBP-1, -2, -4, and -5; data not shown). The molecular mass of $M_r$ = 30 kDa was similar to that reported by other investigators [31, 32]. In none of the saliva samples from either BALB/c or NOD mice was a cross-reactive IGFBP detected. This was substantiated by examination of parotid and submandibular gland cell lysates from BALB/c mice which also failed to detect the presence of binding proteins (Fig. 1, lanes 5 and 6) although antibody to human IGFBP-1 was able to react with the commercially prepared human IGFBP-1 antigen (not shown). Although a faint cross-reactive band appears to be present in the saliva and gland lysates of mice, this band also appeared when blots were incubated only with the second antibody conjugated to alkaline phosphate and thus appears to be non-specific (data not shown). Using constant protein from serum, it was evident that diabetic NOD mice produced more IGFBP-2 than the BALB/c controls (Fig. 1).

To further confirm the lack of IGFBPs in whole saliva, proteins were separated on SDS–polyacrylamide gels, transferred to PVDF membranes under non-reducing conditions,

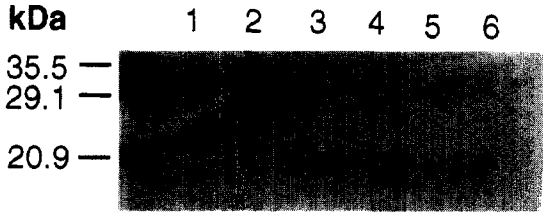


**FIG. 1. Western blot detection of IGFBP-2 with cross-reactive polyclonal antibody to human IGFBP-2. Samples were reduced and denatured prior to electrophoresis on a 10% SDS–polyacrylamide gel and transferred to PVDF membranes. The lanes represent: (1) sera from BALB/c mice; (2) sera from diabetic NOD mice; (3) saliva from BALB/c mice; (4) saliva from diabetic NOD mice; (5) parotid gland lysate from BALB/c mice; and (6) submandibular gland lysate from BALB/c mice. Western blot detection was performed on two separate preparations of N = 5 animals/group. The western blot represents a typical profile for individual samples. The prestained molecular weight standards (Bio-Rad) are: carbonic anhydrase, 35,500; soybean trypsin inhibitor, 29,100; and lysozyme, 20,900.**

and incubated with [$^{125}$I]IGF-I. Using this ligand binding assay, we were able to confirm and expand our observations. Multiple bands were observed in sera from BALB/c and diabetic NOD mice. The $M_r$ = 25–45 kDa was consistent with previously reported results in the literature [31, 32]. As observed in Fig. 1, a protein of approximately 30 kDa was increased in diabetic mice, while there was less binding of radiolabeled ligand for proteins $M_r$ = 25, 40, and 43 kDa (Fig. 2). Densitometric analysis showed that diabetic NOD mice had a 4-fold higher level ($P < 0.01$) of IGFBP-2 than BALB/c mice, while the 25-kDa protein (IGFBP-4) was reduced by 60% ($P < 0.05$). The doublet at $M_r$ = 40–43 kDa is consistent with the polyacrylamide gel mobility of IGFBP-3 and was reduced dramatically in the sera collected from diabetic NOD animals (Fig. 2). The sera of both diabetic NOD and BALB/c mice contain a high molecular weight [$^{125}$I]IGF-I binding protein which may represent aggregated IGFBPs. No binding of radiolabeled ligand was detected in saliva or gland lysates prepared from the mice, which is consistent with the antibody data of Fig. 1.

Saliva and sera from BALB/c mice were incubated with radiolabeled IGF-I and separated on a non-denaturing polyacrylamide gel to detect ligand binding gel shift patterns upon interaction with IGFBPs. Using non-denaturing conditions, a band shift to a higher molecular mass of [$^{125}$I]IGF-I was only detectable after incubation with serum (Fig. 3, lane 3). No observable shift in IGF-I mobility was detected when saliva was used instead of serum (Fig. 3, lane 2).

Without the presence of IGFBPs in saliva, we examined the potential for IGF-I to be absorbed from the gastrointestinal tract and distributed as a biologically active protein to various tissues. Radiolabeled growth factor was administered through the gastrointestinal tract by a gavage technique. As shown in Fig. 4 (A–C), [$^{125}$I]IGF-I was recovered from all tissues examined 30 min following gavage administration. Approximately 8–10% of the radioligand can be found absorbed into the circulation at this time. Parotid and submandibular glands and serum accumulated similar high concentrations of growth factor in the BALB/c and pre-diabetic NOD mice with the other tissues showing less. In contrast, administration of $^{125}$I-labeled IGF-I to diabetic NOD animals showed that the greatest accumulation of growth factor was in the serum. Serum levels of diabetic NOD animals were significantly higher than BALB/c and pre-diabetic NOD mice ($1.9 \times 10^4$ vs $2.5 \times 10^4$ cpm: $P < 0.02$). On the other hand, levels were reduced from approximately 50% (liver and parotid) to 80% (pancreas) in all tissues except the kidney ($P < 0.05$; Fig. 4).

Extraction of [$^{125}$I]IGF-I from the serum, parotid gland, submandibular gland, and pancreas with 1.0 N acetic acid was followed by molecular sieve chromatography on a Sephadex G-50 column [33–35] to determine if [$^{125}$I]IGF in the tissues had been degraded. Greater than 90% of the radiolabeled growth factor was recovered in the soluble material. The soluble tissue extracts were chromatographed on the Sephadex G-50 column and found to co-migrate with

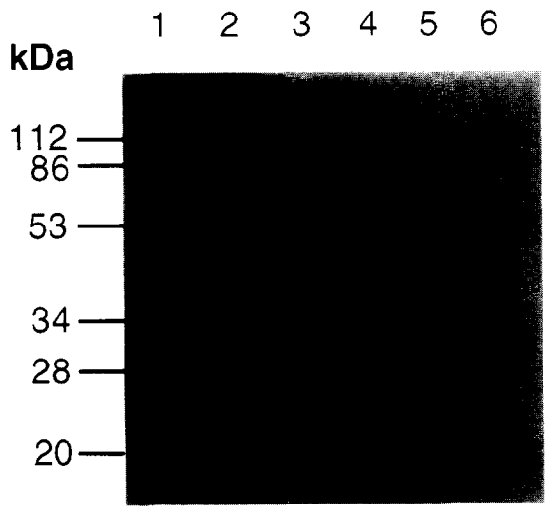


**FIG. 2. Autoradiograph of [$^{125}$I]IGF-I ligand binding to serum, saliva, and gland lysates from BALB/c and diabetic NOD mice. Protein samples were denatured but not reduced with 2-mercaptoethanol prior to electrophoresis on a 10% SDS–polyacrylamide gel and transfer to PVDF membranes. Following blocking, the filters were incubated in TBS containing 0.1% Tween 20, 1% BSA, and [$^{125}$I]IGF-I. Ligand binding was performed on two separate preparations of N = 5 animals/group. The autoradiograph represents a typical profile for individual samples in the experimental groups. All lanes are as in Fig. 1. The prestained molecular weight standards are; phosphorylase B, 112,000; bovine serum albumin, 86,000; ovalbumin, 53,000; carbonic anhydrase, 34,000; soybean trypsin inhibitor, 28,000; and lysozyme, 20,000.**

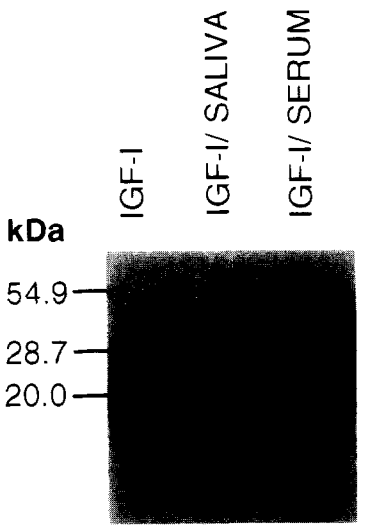


**FIG. 3. Autoradiograph of sera and saliva from BALB/c mice incubated with [$^{125}$I]IGF-I to detect IGFBPs following electrophoretic separation on a nondenaturing 12% polyacrylamide gel. Radiolabeled IGF-I ligand was incubated as per Materials and Methods prior to electrophoresis. The samples were: IGF-I incubated alone; IGF-I with 15 μg of whole BALB/c mouse saliva; and IGF-I incubated with 10 μg BALB/c mouse serum. The binding assay was carried out on three separate occasions with N = 5 saliva or sera samples.**

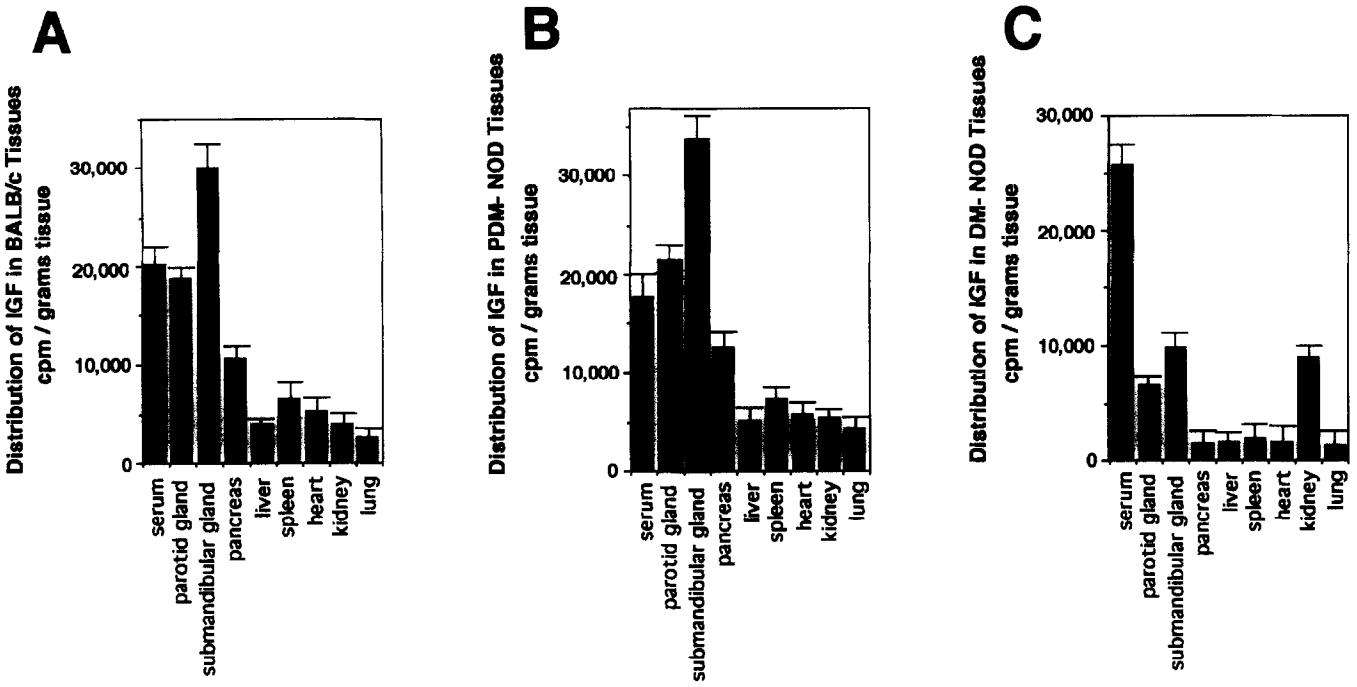


**FIG. 4. Histogram of tissue distribution of [$^{125}$I]IGF-I administered by gastrointestinal tract gavage. Selected tissues were isolated and minced in PBS before counting. Sera values for $^{125}$I-labeled growth factor are expressed as cpm/mL. Values are the means ±SEM in cpm/g tissue wet weight collected at 30 min after radioligand administration (average of N = 4 observations). Tissue distribution is given from BALB/c (panel A), pre-diabetic (PDM; panel B) and diabetic NOD (DM; panel C) animals.**

authentic [$^{125}$I]IGF-I (Fig. 5). There was no evidence of free $^{125}$I or partial degradation products of the growth factor. This column also was able to effectively separate contaminating tissue EGF.

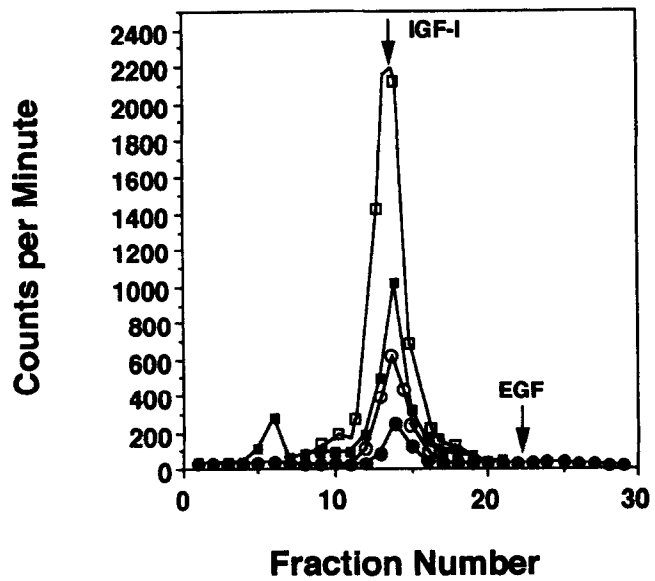


**FIG. 5. Chromatographic analysis of [$^{125}$I]IGF-I extracted from tissues with 1.0 N acetic acid after labeling for 30 min. The originally labeled IGF-I (closed squares) was used to calibrate the column for elution profile of intact growth factor. EGF, also produced by the salivary glands, was chromatographed on the column [33] to demonstrate separation from IGF-I. The samples are parotid gland, open squares; kidney, open circles; pancreas, closed circles.**

The [$^{125}$I]IGF-I extracted from various tissues and purified by chromatography in Fig. 5 was concentrated by lyophilization and subsequently analyzed for retention of the biological property of promoting cell proliferation of tissue culture fibroblasts in the absence of serum. As shown in Fig. 6A, gingival fibroblasts underwent a burst of DNA synthesis and proliferation following the addition of 10% FBS or increasing concentrations of the original IGF-I in MEM. A similar observation was made with different tissue extracts, indicating a retention of biological activity following gastrointestinal tract uptake and distribution. A representative profile is included for parotid gland extracted growth factor assayed at three concentrations (Fig. 6B).

## DISCUSSION

The salivary glands of mice and rats have been shown to produce a wide array of growth factors that are secreted into saliva [5–17]. Research reports from a number of different laboratories have provided conclusive evidence that EGF in saliva contributes to normal tissue homeostasis and promotion of both oral and systemic wound healing [for review, see Refs. 2–5]. Although other growth factors have been identified, their role in oral health has not been assessed. In the case of IGF-I and IGF-II, it has been shown that their tissue specific activity is modulated in part by the presence of a specific class of binding proteins [31, 32]. Thus, we examined saliva for the presence of IGFBPs and found that they are not only absent from saliva but also do not appear

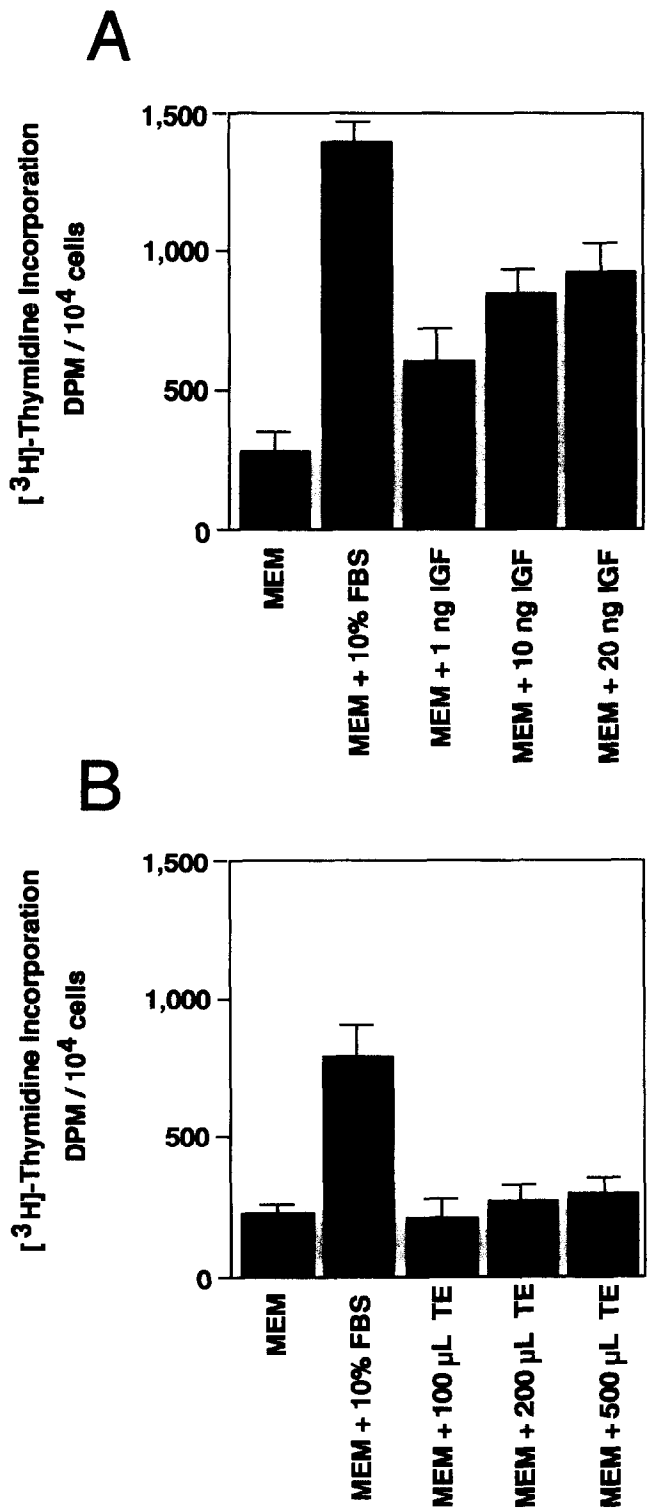


**FIG. 6. Histogram of [$^3$H]thymidine incorporation into DNA following exposure of fibroblasts to growth stimulation by 10% FBS or tissue-extracted [$^{125}$I]IGF-I. Fibroblasts were grown in MEM + 10% FBS split into wells containing MEM and cultured for 24 hr. Growth stimulation was provided by the addition of 10% FBS or different concentrations of pure IGF-I (Amersham) as shown in panel A. In panel B, three concentrations of tissue extracted and chromatographed IGF-I from Figs. 4 and 5 were added to the culture medium. Values are the means ± SEM for 3 wells of cells.**

to be synthesized by the salivary glands of rodents. To demonstrate the consistency of this result, we used standard western blot techniques with a cross-reactive antihuman IGFBP-2 antibody as well as *in vitro* radiolabeled ligand binding assays with saliva, salivary gland lysates, and serum.

Although we found that our commercial antibodies against human IGFBPs were of limited use against mouse proteins, at least one antibody to IGFBP-2 was able to detect a putative binding protein in the sera of mice. Positive interaction by ligand binding assays was established with a series of presumptive IGFBPs in the sera from BALB/c and diabetic NOD mice, which confirmed their presence. The gel migration for the binding proteins was consistent with those reported by others [31, 32, 37]. Interestingly, we noted changes in the concentration of the IGFBPs, in the NOD mouse model for autoimmune type 1 IDDM. An increase in serum concentrations of IGFBP-2 has been noted previously to occur in rats treated with streptozotocin to induce diabetes [31]. Therefore, it would appear that the increase in IGFBP-2 observed here in a model for human type 1 IDDM is consistent with results obtained in other species and models for the disease based on a chemical destruction of the insulin-producing islet cells rather than by autoimmunity [38].

The lack of IGFBPs in saliva was investigated as to whether or not they would influence mucosal uptake of IGF-1 from the gastrointestinal tract. Our observations suggest that radiolabeled IGF-I is able to cross the mucosa of the gastrointestinal tract in a biologically active form and be systemically distributed. Reduced growth and lowered circulating IGF-I concentrations are often observed in uncontrolled IDDM [39, 40]. Although our NOD mice were maintained with insulin supplementation after diabetes onset, we observed elevated levels of IGF-I in the sera isolated from these mice. Tissue distribution of radiolabeled IGF-I was reduced substantially compared with BALB/c controls in all tissues except for kidney.

Several of the IGFBPs are thought to regulate IGF availability and action *in vivo* and *in vitro* [29–32]. The influence of IGFBPs on IGF-I and IGF-II has been reported extensively for the regulation of proliferation and differentiation of bone cells [41]. Mohan *et al.* [31] reported that IGFBP-4 was able to inhibit binding of IGFs to its membrane receptor, while IGFBP-5 increased IGF binding to the cell surface and cell proliferation. It may be possible that IGFBP-2 is able to bind more IGF-I in the serum from NOD mice and prevent its release at the cell surface of the various tissues examined. Alternatively, there may be a generalized decrease in IGF or IGFBP receptors which would fail to bind radiolabeled growth factor and lead to systemic tissue distribution. This lack of significant organ distribution could contribute to the overall reduction in growth and wound healing observed in IDDM.

In summary, while we report that saliva does not contain IGFBPs, saliva derived IGFs can still potentially be absorbed from the oral and gastric mucosa in biologically ac-

tive forms. Furthermore, the tissue distribution in controlled IDDM in the NOD mouse model is reduced, which may ultimately contribute to reduced growth and wound healing potentials.

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