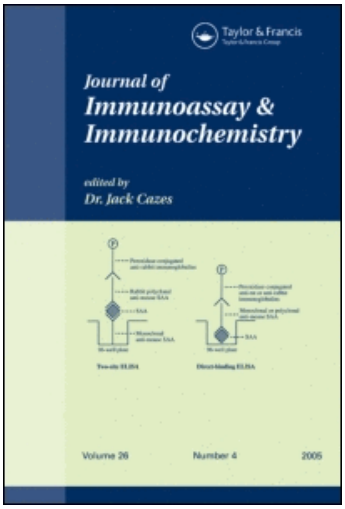

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TRANSFORMING GROWTH FACTOR-α IN HUMAN SUBMANDIBULAR GLAND AND SALIVA

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ABSTRACT

A sensitive sandwich enzyme immunoassay (EIA) for transforming growth factor-α (TGF-α) utilizing a polyclonal antibody that recognizes limited epitopes of both human TGF-α and rat TGF-α in combination with a monoclonal anti-TGF-α IgG_1 galactosidase conjugate was developed. This assay shows no cross-reactivity with human epidermal growth factor. We can quantify the TGF-α level in not only human TGF-α (detection limit: 1 pg/ml), but also rat TGF-α (detection limit: 10 pg/ml) by virtue of cross-reactivity. Employing this assay system, we demonstrated that TGF-α is present in both human submandibular glands and submandibular/sublingual saliva.

KEY WORDS : TGF-α, EGF, EIA, submandibular gland.

INTRODUCTION

Transforming growth factor-α (TGF-α) is a single polypeptide that acts as a mitogen (1) and is a member of the epidermal growth factor (EGF) family of structurally related mitogenic polypeptides (2-4). TGF-α and EGF are derived from membrane-anchored precursors (5), biologically active via their ability to interact with the EGF receptor, and exert a variety of similar biological activities both *in vivo* and *in vitro* (1,6). Therefore, TGF-α has been considered to be the oncofetal counterpart of EGF. The primary sites of synthesis of EGF have been demonstrated to be the salivary gland in human and rat (7). Although TGF-α synthesis has usually been associated with transformed and tumor cells (8), expression of TGF-α mRNA has also been noted in normal tissues (9). Recent studies indicate that TGF-α and/or EGF exhibits neurotrophic effects upon certain populations of neurons in culture and in tissues (10-12). However, there is no consensus about the main source of TGF-α in normal tissues. In order to clarify the role of TGF-α in the mammalian body, a specific, quantitative, and highly sensitive assay for TGF-α is now indispensable. We reported previously an ELISA for the detection of human TGF-α, using monoclonal IgM, polyclonal IgG, and peroxidase-labelled polyclonal antibody (13,14). However, a disadvantage of this assay was its high limit of detection (100 pg/ml), long time incubation for assay (total time: 7 hr), and complex pretreatment for measurement of TGF-α. Further, the assay could not measure rat TGF-α.

In view of these limitations, we developed a two-site sandwich enzyme immunoassay (EIA) for human TGF-α employing monoclonal IgG_1 conjugated with β-D-galactosidase and polyclonal antibodies with defined epitopes. This assay yields quantitative results of high accuracy, and saves time (3 hr for assay) because double-antibody-sandwich-form was performed in the first incubation. Furthermore, this assay can also be utilized to quantify rat TGF-α as a practical procedure.

MATERIALS AND METHODS

Native, fully bioactive human TGF-α was obtained from the culture supernatants of the Seki(C24) human malignant melanoma cell line (15). Phenylmethylsulfonylfluoride (PMSF) and other reagents used were all commercially provided. The polyclonal antibody (PoAb) against human TGF-α (hTGF-α (1-50)) was produced in rabbits (13). The epitope of this PoAb seems to be around the very end of the C terminus, 44-50. Anti-hTGF-α monoclonal antibody (MoAb) was prepared according to a previously published procedure (13). The epitope of MoAb (IgG_1) was shown to reside in ST2, hTGF-α (16-33).

Enzyme immunoassay procedure

The immunoreagents were essentially similar to those described previously (16,17). Briefly, the polystyrene beads of the solid phase with immobilized anti-TGF-α IgG (PoAb) were incubated with both various amounts of standard human TGF-α or samples and the MoAb (anti-TGF-α IgG_1) conjugated with β-D-

galactosidase at 37 °C with vigorous shaking in a final 250 μl of 0.01 M sodium phosphate buffer (pH 7.0)/ 0.3 M NaCl/ 1 mM $MgCl_2$/0.5% gelatin/0.1% BSA/ 0.1% NaN_3 and protease inhibitors (leupeptin, pepstatin, antipain, each 5 μg/ml, and 50 μM PMSF). After 2 h the reaction medium was removed by aspiration, and the beads in each test tube were washed twice with 1 ml of chilled buffer-1 (0.01 M sodium phosphate buffer(pH 7.0)/ 0.1 M NaCl/ 1 mM $MgCl_2$/ 0.1% BSA). The bound enzyme protein was assayed fluorometrically with 4-methyl-umbelliferyl-β-D-galactoside as a substrate. Human EGF was measured by two-site sandwich ELISA utilizing two kinds of monoclonal antibodies, as described previously (18).

Urine, plasma, cerebrospinal fluid, submandibular gland and saliva

Urine from cancer patients (hepatoma, 9 males, mean 54.6 years) and cerebrospinal fluid (CSF) were supplied by Dr. T. Kage (Horinouchi Hospital, Saitama, Japan). Urine (6 males, mean 36.3 years) and plasma (17 males, mean 25.2 years) from healthy donors were collected from volunteers from among our staff. CSF samples (23 males, mean 48.5 years) were obtained by lumbar puncture from control patients without neurological diseases who were receiving operations under lumbar anesthesia. Informed consent for the analysis of urine and CSF were given by each patient. One human submandibular gland was obtained at autopsies within 24 hr after death. Rat submandibular, sublingual and parotid gland were also obtained from Sprague Dawley Rat (male, 10 weeks of age). All samples were stored at

- 80 °C until assayed. Tissue samples were homogenized with 0.25 M sucrose and then centrifuged at 100,000g for 30 min. Protein content was determined by protein dye binding, with bovine serum albumin employed as a standard, by use of a Bio-Rad protein-assay kit (19).

Collection of saliva (unstimulated saliva) was performed with a micro pipet (1 μl) by touching the surface of a drop of saliva just excreted from the orifice of the duct of human or rat, as previously reported (20,21). Control subjects without systemic or salivary gland disease were volunteers from among staff(18 male, mean 24.6 years).

RESULTS

Standardization of EIA

Calibration curves based on the use of known concentrations of human TGF-α and rat TGF-α are shown in Fig.1. The working range of the assay is between 1 and 3 x 10^4 pg/ml for human TGF-α. The detection limit of the assay (2 x blank) is 1 pg/ml for human TGF-α. The EIA also did not detect human EGF up to 1 μg/ml (Fig.1). Plasma samples with low or high TGF-α concentration were analyzed to assess precision (inter- or intra-assay variation). Under similar conditions (the same compositions in the first incubation mixture) as described previously (13), this EIA showed poor reproducibility; that is, the coefficients of variation (CV) ranged from 9.3 to 12.2%. In order to improve this value, we added protease inhibitors (leupeptin, pepstatin, antipain, each at 5 μg/ml, and 50 μM

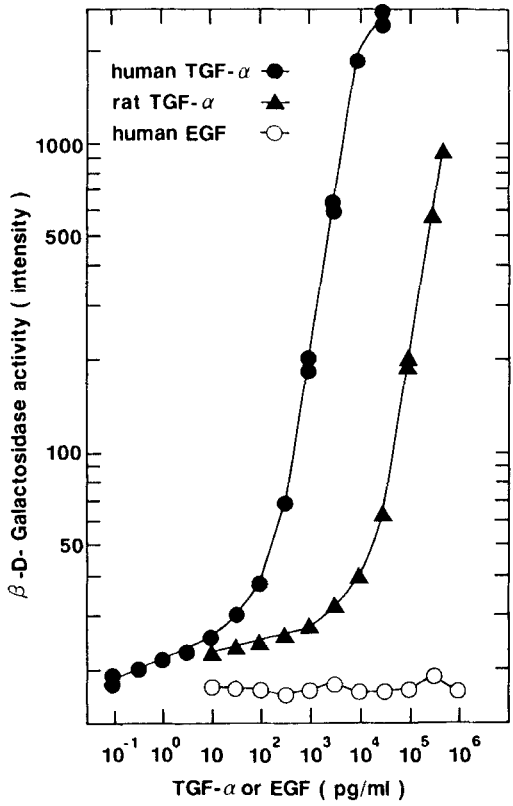

Fig.1. Standard curves of purified TGF- α generated by our sandwich enzyme immunoassay. Human TGF-α (●) or rat TGF-α (▲) was incubated in triplicate with the solid phase (polystyrene beads) containing immobilized anti-human TGF-α IgG. Recombinant human EGF (○) was also tested for detection by this assay.

PMSF) to the first incubation mixture, since it is well known that most human samples contain proteinase activity that can degrade the TGF-α polypeptide. The resulting data revealed much improved precision. All of the CVs were less than 5.6% (data not shown). In addition, the recoveries of human TGF-α added to plasma were 93.5-104%, when TGF-α at three different levels (11.3, 61.3, and 261.3 pg/ml) was added to three plasma samples (20 μl) containing 68.7-288.7 pg/ml of TGF-α. However, without the addition of protease inhibitors, the recovery varied to a greater extent (86-125%, Table 1). This assay system save a time because double-antibody-sandwich form was performed in first incubation. Total assay time was within 3 hr. Using samples from 10 to 2000 pg of TGF-α (n = 17), we compared the present EIA with the previous ELISA system (13). The assays correlated closely ($r^2 = 0.97$).

Furthermore, this assay could detect rat TGF-α due to the cross-reactivity of the antibodies. The epitope recognized by the primary-capture antibody, anti-TGF-α IgG, is identical between human and rat TGF-α (13). A further confirmation of the epitope for monoclonal IgG_1 was shown in that this IgG_1 seemed to distinguish human TGF-α from rat TGF-α since aspartic acid-28 in human TGF-α was the only amino acid substituted in the ST2 region of rat TGF-α (13). The standard curve for the rat molecular paralleled that for the human one, as shown in Fig.1, and the cross-reactivity of rat TGF-α was approximately 2% of the human TGF-α reactivity. The assay range for rat TGF-α was 0.01-600 ng/ml, which is practical measurement range; and the

TABLE 1

Recovery Test of TGF-α EIA System Using Human Plasma

	Added	Calculation (pg/ml)	Found (pg/ml)	
			No addition	+ Protease inhibitors
Sample 1	0	-	68.7	68.7
	11.3	80	68.8 (86)	82.8 (104)
	61.3	130	162.5 (125)	121.5 (93.5)
	261.3	330	336.3 (102)	328.0 (99.4)
Sample 1	0	-	137.3	137.3
	11.3	150	172.3 (115)	141.7 (94.5)
	61.3	200	213.5 (107)	208.3 (104)
	261.3	400	390.0 (97.5)	390.5 (97.6)
Sample 1	0	-	288.7	288.7
	11.3	300	311.5 (104)	292.3 (97.4)
	61.3	350	341.2 (97.5)	358.8 (103)
	261.3	550	565.6 (103)	564.7 (103)

Protease inhibitors: leupeptin, pepstatin, and antipain, (each 5 μg/ml); and PMSF (50 μM)

detection limit of the assay is 10 pg/ml. The CV of intra- or interassay variation was less than 11.4% (Table 2).

Determination of TGF-α in human and rat samples

Due to the low sensitivity of previous assay systems (13,14, 22-24), all detection systems for TGF-α required both pretreatment and preconcentration of samples, in advance. The present EIA overcomes these problems. All samples can be

TABLE 2

Intra- and Interassay Variability of TGF-α EIA System for Rat TGF-α

Sample	TGF-α (pg/ml)	Coefficient of Variation (CV %)
Interassay		
1	71 ± 5.9	8.3
2	510 ± 27	5.2
3	3530 ± 135	3.8
Intrasaay		
1	71 ± 8.1	11.4
2	490 ± 44	8.9
3	3510 ± 227	6.5

Mean ± S.E.M. n = 4

tested directly in this EIA, and it can measure the low level of TGF-α in control human urine, CSF and plasma (Table 3). These results are consistent with those reported previously (13,24).

When the pooled sample of whole human saliva was assigned for immunoreactive TGF-α protein using a present EIA, logit transformation of the displacement data generated lines with slopes that were not significantly different from the slope of the line generated by authentic human TGF-α, indicating that

TABLE 3

Determination of TGF-α in Human Samples

Human sample	Sample number	TGF-α (pg/ml)
Plasma control	17	13.6 ± 8.8
Cerebrospinal fluid control	23	1.3 ± 0.5
Urine control	6	2.6 ± 0.5
hepatoma	9	43.3 ± 4.0
Human Seki (C24) malignant melanoma	1	4800
Human Kato III adenocarcinoma, stomach	1	560

Mean ± S.E.M.

presence of immunoreactive TGF-α protein in the saliva samples (data not shown). The calculated levels of immunoreactive TGF-α protein in human samples are shown in Table 3 and 4. We found TGF-α in both human saliva and submandibular gland (Table 4). The concentration of TGF-α was much lower than the EGF in the samples tested. The EGF content was 6-fold higher than the TGF-α content in whole saliva. EGF is known to be abundant in submandibular gland, while immunoreactive TGF-α in the submandibular gland was detected at less than 1% of

TABLE 4

TGF-α and EGF Concentration in Human Samples

Human	TGF-α (pg/ml)	EGF (pg/ml)
Whole saliva (18)	10.8 ± 3.9	65.4 ± 15.4
Parotid saliva (18)	7.2 ± 2.5	48.4 ± 11.8
Submandibular and sublingual saliva (18)	24.8 ± 7.2	150 ± 23
Submandibular gland (1)	5.9 pg/mg prot.	482 pg/mg prot.

Mean ± S.E.M. prot., protein. Number of cases are shown in parentheses.

the level of EGF. When we subjected the extracts from human submandibular gland to SDS-PAGE and Western blotting using anti-TGF-α IgG, the samples gave a high-density 6-kDa band, which corresponded to the same relative molecular mass as authentic human TGF-α (data not shown). These data indicate, at least in a preliminary way, that TGF-α is present in human submandibular gland and saliva.

The results of assays conducted an various rat tissues are summarized in Table 5. The concentration of TGF-α in rat saliva was 2-3 fold higher than the level of growth factor assayed in human saliva. We also found TGF-α in all salivary gland extracts.

TABLE 5

TGF-α and EGF Concentration in Rat Samples

Rat	TGF-α (pg/ml)	EGF (pg/ml)
Parotid saliva (10)	17.6 ± 2.1	71.6 ± 10.5
Submandibular and sublingual saliva (10)	88.4 ± 17.9	234 ± 42
Submandibular gland (1)	39 pg/mg prot.	662 pg/mg prot.
Sublingual gland (1)	27 pg/mg prot.	175 pg/mg prot.
Parotid gland (1)	12 pg/mg prot.	460 pg/mg prot.

Mean ± S.E.M. prot.: protein. Number of cases are shown in parentheses.

DISCUSSION

This EIA offers three main advantages: First, it is much more sensitive than previous RIA or ELISA for TGF-α (13,22,23). The suitable choice for antibodies with limited epitope has made it possible to determine 1 pg/ml of TGF-α in human samples. Secondly, it can save time since neither pretreatment nor preconcentration is needed, for example, dialysis against 1 M acetic acid, lyophilization, and Sep-Pak C18 chromatography (13,22-24). Finally, this EIA is applicable to determine the concentration of rat TGF-α as a practical procedure that was

previously unavailable. The most striking finding is that TGF-α itself is present in human submandibular gland and saliva. Surprisingly, a relatively high level of TGF-α is also present in rat submandibular glands and saliva. It is well known that the main source of endogenous EGF is the submandibular gland, and our data indicate that TGF-α may co-locate with EGF in salivary gland. Given that TGF-α and EGF bind to the same receptor and have structural and biological function similarities, it is tempting to speculate that TGF-α is the minor endogenous ligand for the EGF receptor in the salivary gland.

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Abbreviations: TGF-α, transforming growth factor-α; EGF, epidermal growth factor; PMSF, phenylmethylsulfonylfluoride ; CV, coefficient of variation; MoAb, monoclonal antibody; PoAb, polyclonal antibody.

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