Human Transforming Growth Factor-β3: Recombinant Expression, Purification, and Biological Activities in Comparison with Transforming Growth Factors-β1 and -β2

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Recent cDNA characterization has predicted the existence of a new member of the transforming growth factor family, transforming growth factor-β3 (TGFβ3). However, nothing is known about the biological activities of the TGFβ3 protein, since it has not been purified from any natural sources. We report here the recombinant expression in mammalian cells and the purification to apparent homogeneity of human TGFβ3. The TGFβ3 was evaluated in comparison with purified TGFβ1 and TGFβ2 in several assays for its effects on stimulation or inhibition of proliferation of mammalian cells. These analyses revealed that TGFβ3 exerts activities similar to the two other TGFβ species, but that there are distinct differences in potencies between the different TGFβ forms depending on the cell type and assay used. (Molecular Endocrinology 3: 1977–1986, 1989)

INTRODUCTION

Transforming growth factor-β (TGFβ) was first identified as a factor that could stimulate some rodent fibroblast cell lines to grow in soft agar (1, 2). Subsequently, it became clear that TGFβ has a variety of biological activities in many distinct cell systems. While it can induce the proliferation of fibroblasts in monolayer, it is also a potent inhibitor of cell growth for many cell types, a modulator of differentiation, and a stimulator of extracellular matrix production and deposition (3–5). A vast majority of the biological and structural characterization studies have been performed using TGFβ purified from human platelets (6, 7). It was the polypeptide structure of this TGFβ species that was first determined from cDNA cloning (8). This TGFβ, which we now call TGFβ1, is synthesized as a 390-amino acid precursor from which the C-terminal 112-amino acid monomer is cleaved. Biologically active TGFβ is a dimer of these two identical monomers. Cells secrete TGFβ as a latent complex, due to a noncovalent interaction of the mature TGFβ dimer and a dimer of the N-terminal precursor segments (9, 10). Conversion of this latent TGFβ complex into the active molecule takes place after treatment with acid or proteases such as plasmin (11).

It has recently been established that there are several species of TGFβ, coded for by different genes. cDNAs for the structurally related TGFβ2 (12–15) and TGFβ3 have been derived from several mammalian sources (16–18), while TGFβ4 cDNAs have as yet only been isolated from chicken cells (19). Comparison of the human TGFβ1, -β2, and -β3 precursor cDNAs reveals an amino acid sequence identity of about 75–80% within the mature peptide sequences, while the rest of the precursor sequence is only 25–35% conserved (16, 18).

Examination of the expression of the different TGFβ species indicates that most cell lines express TGFβ1 mRNA, while many cell lines of mesenchymal origin contain TGFβ3 mRNA (16). The various species of TGFβ are frequently coexpressed in cultured cells. All three TGFβ mRNA species are also detected during mouse development, and their levels increase with the age of the embryo (15, 19). *In situ* hybridization studies showed that TGFβ1 mRNAs are present predominantly in hematopoietic cells, such as in bone marrow and in developing fetal liver (21, 22), while TGFβ2 mRNA is mainly expressed in developing bone and in the mesenchymal layers adjacent to major epithelia such as the dermis (23). Northern hybridizations with RNA from various adult mouse tissues showed predominant

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expression of TGFβ1 mRNA in spleen, lung, and placenta, which may be due to high levels of expression by hematopoietic cells and macrophages. TGFβ2 and -β3 mRNAs are present in various other tissues, such as adipose tissue, lung, testis, placenta, and the male submaxillary gland (15, 19). These data, thus, indicate that there is differential expression *in vivo* of all three TGFβ species depending on the tissue and cell type. The differential expression and regulation of multiple TGFβ species suggest differences in biological activities and functions.

Some data are available about the comparative bioactivities of TGFβ2 from porcine platelets with human and porcine TGFβ1. These two TGFβ species are equally active in several assays (24–29), but there are some quantitative differences in some systems. Most striking is that TGFβ1 is 60-fold more potent than TGFβ2 in inhibition of DNA synthesis in endothelial cells (26) and about 100-fold more potent in its ability to inhibit interleukin-3-stimulated proliferation of a murine hematopoietic cell line (30). Remarkably, TGFβ2, but not TGFβ1, demonstrated activity in a *Xenopus* mesoderm induction assay (31). In contrast, the biological activities of TGFβ3 have not been determined due to the lack of purified TGFβ3 from natural sources.

In this study we have expressed the human TGFβ3 cDNA in mammalian cells and have purified the mature form of recombinant TGFβ3. The biological activities of TGFβ3 compared to those of TGFβ1 and -β2 were determined in several assays for stimulation and inhibition of proliferation. This analysis demonstrated that all three TGFβ species stimulate or inhibit cell proliferation depending on the cell type, but that there were differences in potencies among TGFβ3, -β2, and -β1.

RESULTS

Recombinant Expression of Human TGFβ3

Two expression vectors were constructed in order to obtain a high level of expression of human TGFβ3. Both plasmids were identical in all elements, with the exception of the TGFβ3 precursor cDNA transcription unit. The promoter, derived from cytomegalovirus, is separated from the cDNA insert by a short intron. The cDNA sequence is followed by a DNA segment from the SV40 late region, which contains the transcriptional termination and polyadenylation signals. The first expression plasmid, pRK5–19RR (Fig. 1), incorporated in its transcription unit the cDNA sequence coding for the porcine TGFβ3 precursor (16), modified to express human mature TGFβ3. This modification was introduced by site-directed mutagenesis of two codons in the sequence coding for the C-terininal porcine mature TGFβ3, such that it encoded the human mature TGFβ3 polypeptide (16). This approach was used because of difficulties in isolating cDNAs coding for the N-terminus of the human TGFβ3 precursor. Also deleted was the entire 5′ untranslated region, including the three ATGs preceding

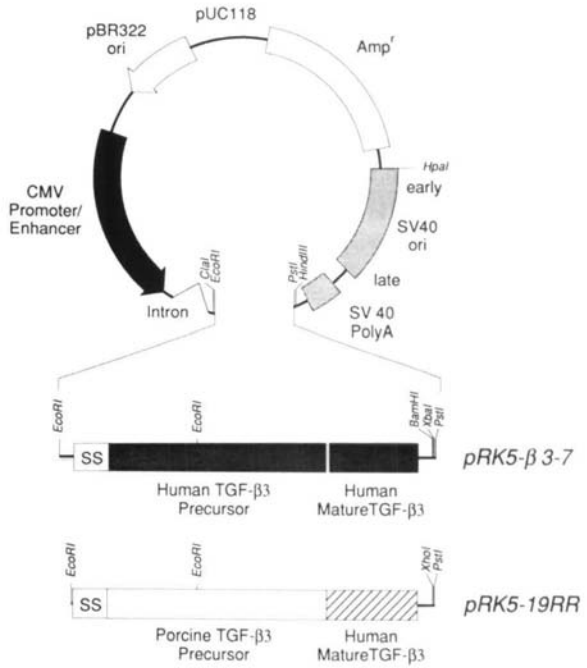

Fig. 1. Schematic Diagram of the TGFβ3 Expression Plasmids pRK5–19RR and pRK5–β3–7

The CMV promoter segment with the direction of transcription is shown as a *heavy black arrow*, and the segments derived from SV40 are shown as *shaded boxes*. The two TGFβ3 cDNA transcription units, corresponding to each plasmid, are shown with some flanking restriction sites. SS, Signal sequence.

the proposed initiation codon, in order to avoid a decreased translational efficiency (32). The other TGFβ3 expression vector, pRK5-β3–7 (Fig. 1), contained the full-size human TGFβ3 precursor-coding sequence (16, 18). The segment of the 5′ untranslated region that contained the upstream ATG triplets was also removed in pRK5-β3–7.

Each TGFβ expression vector was individually cotransfected with the plasmid pSV-dhfr containing a dihydrofolate reductase (dhfr) transcription unit into dhfr-deficient CHO cells. The transfected clones that had acquired a dhfr-positive phenotype, as determined by their ability to grow in a selective medium, were assayed for their secretion of TGFβ. Several cell lines tranfected with pRK5–19RR were further treated with increasing methotrexate concentrations in order to amplify the number of integrated TGFβ3 transcription units and, thus, enhance the TGFβ3 expression levels. One of these cell lines resistant to 1600 nM methotrexate secreted 2 μg TGFβ3/ml·48 h, as determined by the TGFβ bioassay (see *Materials and Methods*). Quantitation by TGFβ radioreceptor assay indicated a 3-fold lower concentration, presumably due to a higher specific activity of TGFβ3 than of TGFβ1 in this assay (see below). Chinese hamster ovarian (CHO) cells transfected with pRK5-β3–7 were not treated with methotrexate. One of these cell lines secreted 30 ng TGFβ/ml·48 h, as determined by this bioassay. The levels of

TGFβ activity in the medium were assessed in this TGFβ bioassay with or without acid treatment. The result indicated that treatment with acid was required for biological activity similarly to TGFβ1 and -β2 (33, 34), thus suggesting that TGFβ3 is made in an inactive latent form which is activated after acid treatment (data not shown).

Purification of Human TGFβ3

A CHO cell line, transfected with pRK5–19RR and amplified in the presence of 1600 nM methotrexate, was used as cell source for the purification of TGFβ3. Confluent cells were incubated with serum-free medium for 2 days, and this conditioned medium was used as starting material for the purification. The purification scheme consisted consecutively of dialysis against acetic acid, lyophilization, gel filtration on Sephacryl S-200, cation exchange chromatography on CM-52 cellulose, and hydrophobic HPLC on a C18 matrix. The degree of purification after each chromatogrraphic step is illustrated in Fig. 2. Extensive dialysis not only resulted in the acid activation of TGFβ3, but also removed many peptides that were smaller than 12 kD and precipitated acid-insoluble proteins. The acid dialysis and gel filtration steps yielded a substantial degree of purity, yet two additional chromatographic steps were needed to remove the remaining contaminant proteins. The TGFβ3 eluted from the C18 HPLC matrix at an acetonitrile concentration of about 45%, while TGFβ1 and -β2 eluted at 38% and 41% acetonitrile, respectively (our unpublished data). We were usually unable to recover all TGFβ3 in a totally pure form after this last chromatography step, since the elution profile of some contaminant proteins partially overlapped the TGFβ3 elution peak. Yet, some fractions contained only the TGFβ3 polypeptide, as assessed by silver-stained polyacrylamide gels (Fig. 2). Starting from 600 ml conditioned medium, about 20 μg TGFβ3 were obtained in a highly purified form (Fig. 3). This TGFβ3 was used for evaluation of the biological activity.

The purity and identity of recombinant TGFβ3 were assessed using several criteria. Analysis of the amino acid composition data yielded a composition that agreed with the predicted polypeptide sequence of human TGFβ3 (data not shown). In addition, direct sequencing of the purified polypeptide resulted in a 20-amino acid N-terminal sequence in agreement with the predicted N-terminus of human TGFβ3 (data not shown). This analysis indicates that the mature TGFβ3 is proteolytically cleaved from the precursor after the multibasic sequence, as was predicted in analogy with TGFβ1 and TGFβ2 (16, 18). Analytical gel electrophoresis followed by silver staining showed a single polypeptide band with an apparent mol wt of 25 kD under nonreducing conditions or of 12 kD when reduced (Fig. 3). No other protein bands were detected. The apparent mol wt values are similar to those of TGFβ1 and -β2 and reflect the fact that this growth factor is a disulfide-linked homodimer of 112 amino acids. However, TGFβ3 has a somewhat lower mobility than TGFβ1 when nonreduced and migrates slightly faster than TGFβ1 after reduction. These differences in mobility are presumably due to the differences in polypeptide sequence and secondary structure, which affect the migration in the polyacrylamide matrix.

Biological Activities of TGFβ3

The biological activities of TGFβ3 were evaluated in several assays of cell proliferation. The recombinant human TGFβ3 was tested in parallel with platelet-derived or recombinant human TGFβ1 and with porcine platelet-derived TGFβ2. All TGFβ species were free of

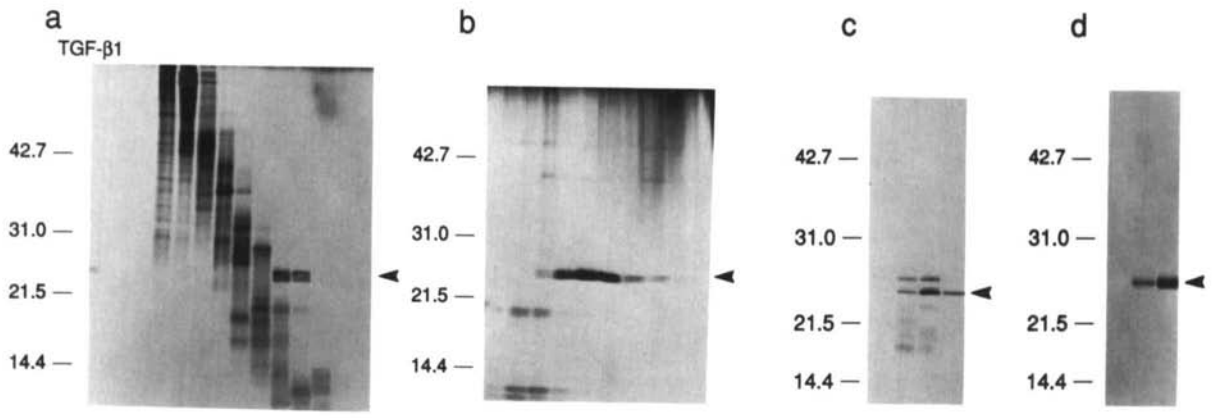

Fig. 2. Gel Electrophoretic Analysis of the Column Profiles during TGFβ3 Purification

The protein bands were visualized by silver staining. The positions of the mol wt markers are shown to the *left* of each panel. The analytical samples were run without reduction. The *arrow to the right* of each panel shows the position of TGFβ3. a, Sepharose G-200 gel filtration profile. Every second column fraction was analyzed on gel. The sample in the *left lane* shows purified human TGFβ1. b, Elution profile from the CM-52 column. c and d, C18 HPLC elution. Only the TGFβ3 peak fractions are shown. c shows a worst case in which only the fraction corresponding to the right lane contained TGFβ3 free of detectable contaminants. d shows a best case in which most of the TGFβ3 was recovered in a highly purified state.

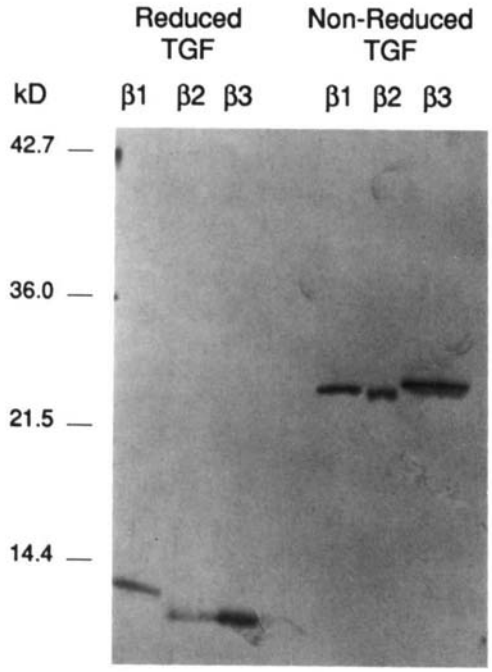

Fig. 3. Electrophoretic Analysis of Purified TGFβ_3 Compared with TGFβ1 and -β2 with or without Reduction before Electrophoresis

The bands were visualized by silver staining.

detectable contaminants, and their concentrations were determined by amino acid analysis. All experiments were performed at least twice in triplicate, and the results from multiple analyses were in agreement.

Before determining the biological activities of TGFβ3, we evaluated its binding to the TGFβ receptors. Using AKR-2B (clone 84A) cells as target cells, TGFβ3 was able to compete with ^{125}I-labeled TGFβ1 for binding to the receptors, as were TGFβ1 and TGFβ2. Some quantitative differences were revealed, indicating that TGFβ1 was more effective in displacing [^{125}I]TGFβ1 than TGFβ2 or -β3 (Fig. 4).

TGFβ1, -β2, and -β3 were also evaluated for their ability to stimulate proliferation of AKR-2B fibroblasts. [^{3}H]Thymidine incorporation was used as a measure of DNA synthesis in these cells. TGFβ3 and -β2 are equally active and are significantly more active than TGFβ1 in stimulating these cells (Fig. 5). While the same maximal response is elicited, TGFβ2 and -β3 appear to be 5- to 10-fold more potent than TGFβ1. It is interesting that the slope of the dose-response curve is steeper for TGFβ1 than for TGFβ2 and -β3.

The designation TGF was originally based on the ability of the growth factor to induce anchorage-independent growth of some cells (1, 2). We, therefore, compared TGFβ1, -β2, and -β3 for their ability to induce the formation of colonies in soft agar, using the AKR-2B (clone 84A) fibroblast cell line. TGFβ1 has previously been shown to induce anchorage independence of these cells in the absence of exogenous epidermal growth factor (EGF) or TGFα (1). All three TGFβ species

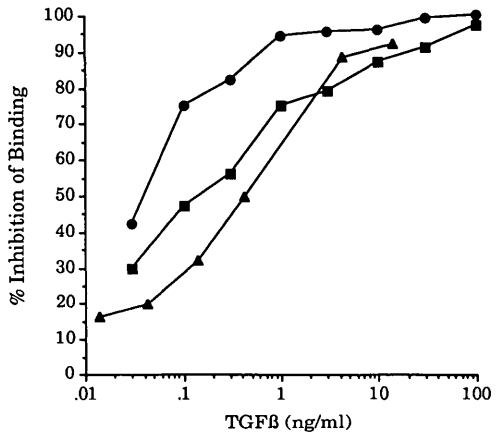

Fig. 4. Competition of [^{125}I]TGFβ1 Binding to AKR-2B Cells Using TGFβ1, -β2, and -β3

TGFβ1 (●), TGFβ2 (■), and TGFβ3 (▲) were coincubated with [^{125}I]TGFβ1 for 2 h at room temperature. Values are expressed as percent inhibition of binding. Each *data point* represents the mean of triplicate determinations. The SEM was less than 10% for each data point.

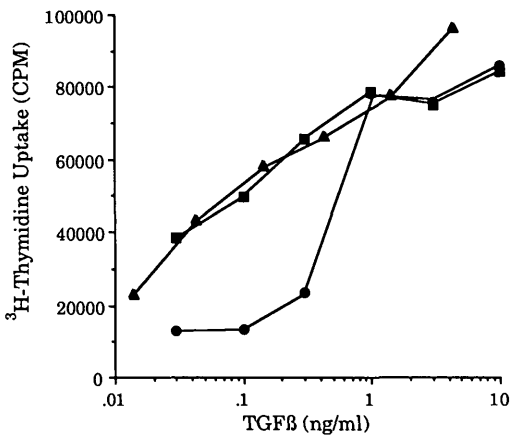

Fig. 5. Stimulation of AKR-2B Cell DNA Synthesis

Varying amounts of TGFβ1 (●), TGFβ2 (■), and TGFβ3 (▲) were added to quiescent AKR-2B cells. Values are expressed as counts per min of [^{3}H]thymidine incorporated into trichloroacetic acid-insoluble counts per min. Each *data point* represents the mean of triplicate determinations. The SEM was less than 10% for each data point.

were able to induce anchorage independence in a similar way (Fig. 6). There are differences in potencies, with TGFβ2 being more active than TGFβ3 and -β1.

It has been recognized over the past few years that TGFβ is a potent growth inhibitor for many normal and transformed cells. The growth inhibition by TGFβ3 was tested in several cell systems. Treatment of mink lung epithelial cell line CCL64 with increasing concentrations of TGFβ1, -β2, or -β3 resulted in an inhibition of DNA synthesis by each TGFβ type, but, again, there were some significant differences in potencies. TGFβ2 and -β3 were equally active, but both were more potent than TGFβ1 in this assay (Fig. 7).

We and others have previously shown that TGFβ1

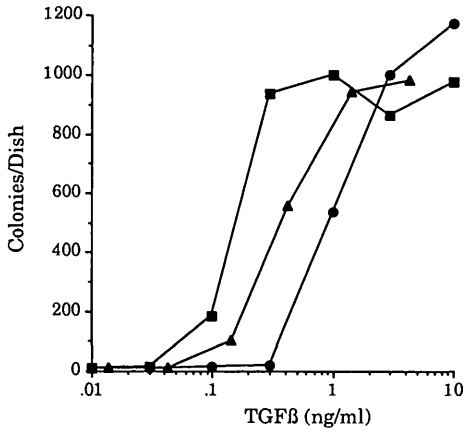

Fig. 6. Stimulation of AKR-2B (Clone 84A) Cell Growth in Soft Agar

Varying amounts of TGFβ1 (●), TGFβ2 (■), and TGFβ3 (▲) were incubated with AKR-2B (clone 84A) cells for 7–10 days. Values represent the number of colonies of cells that grew on each dish per condition. Each value represents the mean of two to four determinations. The SEM was less than 10% for each data point.

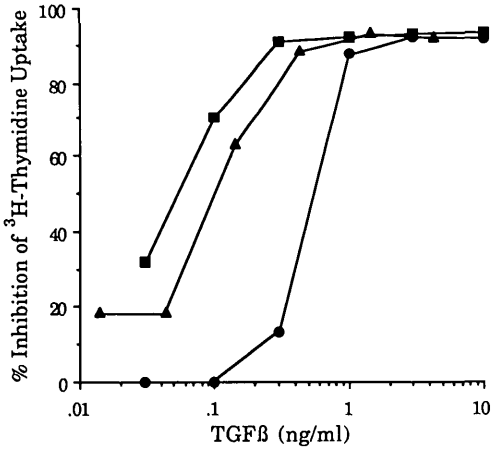

Fig. 7. Inhibition of CCL-64 Cell DNA Synthesis

Varying amounts of TGFβ1 (●), TGFβ2 (■), and TGFβ3 (▲) were added to proliferating CCl-64 cells. Cells were pulsed with [^{3}H]thymidine 23–25 h later. Each value represents the percent inhibition of DNA synthesis, as determined by the decrease in trichloroacetic acid-insoluble material present after the pulse compared to that in untreated cells. Each *data point* represents the mean of triplicate determinations. The SEM was less than 10% for each data point.

and -β2 inhibit the proliferation of the mammary carcinoma cell line MCF-7. The relative inhibitory effects of the three TGFβ species were measured by counting the number of cells in culture after 5 days of treatment. As shown in Fig. 8, TGFβ1 is about 10-fold more active than TGFβ2 in this system, while TGFβ3 has an intermediate potency. Another manimary carcinoma cell line, T-47D, is not affected in its growth by TGFβ1, -β2 or -β3 (data not shown).

Finally, TGFβ1, -β2, and -β3 were tested for their

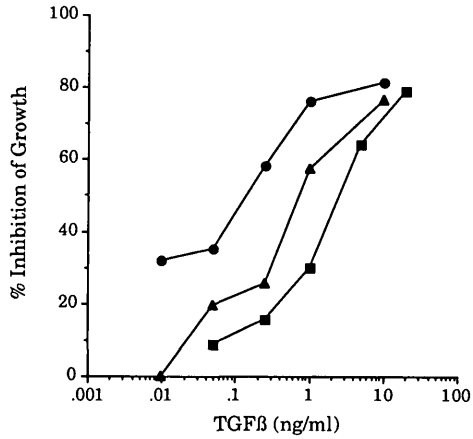

Fig. 8. Inhibition of MCF-7 Cell Growth in Culture

Varying amounts of TGFβ1 (●), TGFβ2 (■), and TGFβ3 (▲) were added to proliferating MCF-7 cells on days 0 and 3. Cells were harvested and counted on day 5. The values represent the decrease in total number of cells compared to that in untreated MCF-7 cells. Each value represents the mean of three determinations. The SEM was less than 10% for each data point.

effects on the proliferation of the mouse keratinocyte cell line BALB/MK and of primary cultures of human keratinocytes. All three TGFβ species inhibited DNA synthesis of both mouse and human keratinocytes (Fig. 9). In both cell systems TGFβ3 appeared more potent than TGFβ2 and -β1. As previously observed with the stimulation of DNA synthesis in AKR-2B cells, the slope of the dose-response curve was steeper with TGFβ1 than with TGFβ2 and -β3.

Comparison of the effects of TGFβ3 with those of the two other TGFβ species in these biological systems thus indicates that TGFβ3 is fully active and elicits growth stimulatory and inhibitory effects similar to TGFβ1 or -β2, but that there are some differences in potencies between the different TGFβ species.

DISCUSSION

Recent evidence has indicated that at least three different TGFβ species, each encoded as the C-terminal segment of very different precursors, are synthesized by mammalian cells. Northern hybridizations have revealed that these three species are differentially expressed in tissues and organs in the adult mouse, suggesting differences in the physiological roles of these TGFβ species (15, 19). Most biological studies on TGFβ have been performed with TGFβ1, due to its relative abundance in blood platelets and since the cDNA encoding TGFβ1 was the first to be isolated and characterized. In contrast, only a few recent studies have compared the activities of TGFβ1 and -β2 and have pointed out many similarities, but also some distinct differences, in their biological activities. There is

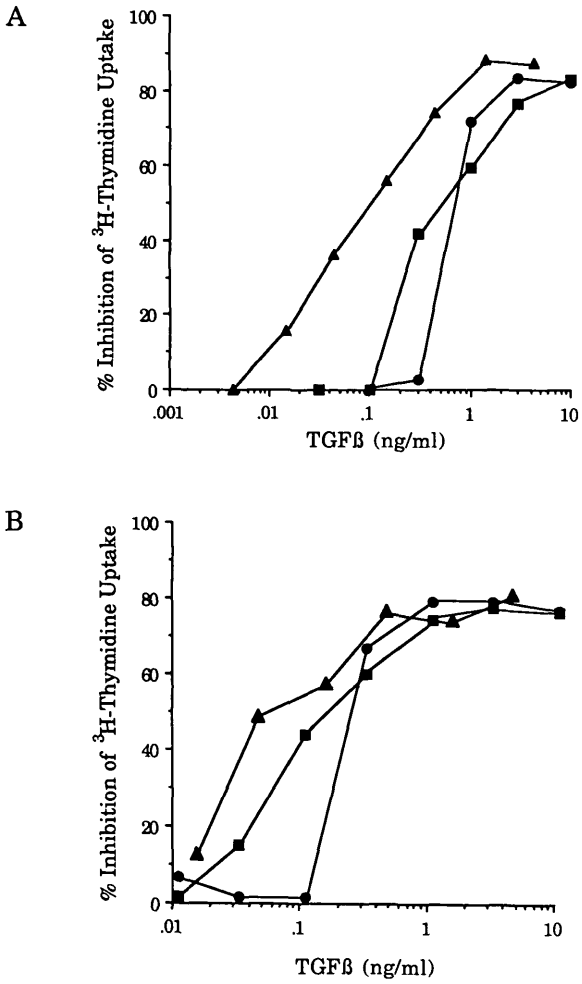

Fig. 9. Inhibition of DNA Synthesis in Keratinocytes

Varying amounts of TGFβ1 (●), TGFβ2 (■), and TGFβ3 (▲) were added to proliferating keratinocytes. [^{3}H]Thymidine was added to the cells 24 h after TGFβ treatment. The trichloroacetic acid-insoluble counts per min were determined after 6 h. Each value represents the percent inhibition of DNA synthesis, as determined by the decrease in trichloroacetic acid-insoluble material present after the pulse compared to that in untreated cells. A, BALB/c mouse keratinocytes. B, Human keratinocytes. Each value represents the mean of two to four determinations. The SEM was less than 10% for each data point.

no information on the biological activities of TGFβ3 due to its lack of availability from natural sources.

In this report we have documented the recombinant expression of human TGFβ3 in mammalian cells and its purification. The biological properties of purified TGFβ1, -β2, and -β3 were compared in several assays, and it was found that TGFβ3 possesses similar activities as TGFβ1 and -β2, but that there are distinct differences in potencies. Competition experiments of the different TGFβ species using radiolabeled TGFβ1 indicate that TGFβ3 binds to the same receptors as TGFβ1, as was expected on the basis of the structural similarity, and that, at least on the AKR-2B cells used, TGFβ2 or -β3 are less efficient in receptor competition with [^{125}I] TGFβ1 than TGFβ1. The basis for these quantitative differences is unknown, but they are presumably due to different steric interactions. It has been previously shown that when related growth factors interact with a common receptor, differences in receptor binding can be observed. As an example, human TGFα competes with a 2-fold lower efficiency than EGF for binding to the human receptor (35), but is 100-fold more efficient than EGF in binding to the chicken receptor (36).

The results presented in this report show that TGFβ3 exerts similar activities as TGFβ1 and TGFβ2, but that the relative potencies of the different TGFβ species differ depending on the cell system and the assay used. For instance, TGFβ1 is more effective than TGFβ2 or -β3 in inhibiting the proliferation of MCF-7 cells, while TGFβ3 is more potent than TGFβ1 or -β2 in inhibiting the DNA synthesis of human and mouse keratinocytes. TGFβ2 and -β3 behaved similarly to each other, but quite distinct from TGFβ1 in two assays: the inhibition of DNA synthesis in CCL 64 cells and the stimulation of DNA synthesis in AKR-2B cells. In both cell systems TGFβ1 was significantly less active than TGFβ2 and -β3. It is also striking that in both these assays and in the inhibition of DNA synthesis of human and mouse keratinocytes, the slope of the TGFβ1 dose-response curve was significantly steeper than with TGFβ2 or TGFβ3. Our results, thus, indicate that the TGFβ3 precursor cDNA encodes a biologically active TGFβ species and that there are distinct differences in potencies among the three different TGFβ species, which vary depending on the cells and assays used.

It is important to emphasize the need to use purified factors in these measurements of biological activities and to compare the relative potencies on a weight or molar basis. Obviously, the comparative determination of the biological effects needs to be expanded to various other systems and parameters before we can understand the implications for the differential physiological roles of the different TGFβ species *in vivo*. Nevertheless, it is striking that TGFβ3 is more potent than the two other TGFβ species for keratinocytes, a fact that may be of relevance for the physiology of TGFβ3 *in vivo*. These studies will have to be paralleled by detailed characterization of the sites of synthesis of the different TGFβ species. The initial studies have revealed major sites of synthesis for each TGFβ (15, 19), although further analysis using histological methods is certainly required.

The expression of multiple TGFβ species *in vivo* raises the question of the biological relevance of this complexity. One should assume that there are differential mechanisms for discrimination between the effects of the expression of each individual TGFβ species. There are clearly differences not only in the sites of their synthesis but also in the regulation of expression of the different TGFβ species within the individual cell. As an example, the mRNA levels for each TGFβ type are clearly differentially regulated by TGFβ1 and TGFβ2 (37). Also, TGFβ2 and -β3 mRNA levels, but not the TGFβ1 mRNA level, are decreased by estrogen in some breast carcinoma cell lines (38). Another major differ-

ence in the regulation of the activity of the different TGFβ species may result from the very different nature of the precursor sequences (16, 18). As a consequence, it is likely that there are major differences between the latent complexes of the individual TGFβ species, which may necessitate the availability of different mechanisms of proteolytic activation of the complex into the corresponding mature TGFβ type. Finally, there are differences in potencies among the different TGFβ forms depending on the cell type for TGFβ1, -β2, and -β3, as illustrated in this report and in other reports for TGFβ1 and -β2. It is as yet unknown how the observed differences in potencies correlate with the relative binding patterns of the individual TGFβ species to the corresponding receptors. Characterization of the interactions at the molecular level will be needed to understand the molecular basis of the differential activities of the different TGFβ species.

MATERIALS AND METHODS

Plasmid Constructions

The two plasmids used for mammalian expression of the TGFβ3 precursor are both derived from the vector pRK5 (39). Proceeding in a clockwise orientation, this plasmid contains the cytomegalovirus (CMV) promoter and enhancer sequences. This segment is followed by a small intron located between the initiation site of transcription and a polylinker site into which cDNA segments can be inserted. This polylinker is, in turn, followed by a segment of SV40 DNA (originally a *Kpn*I-*Hin*dIII fragment) (40) that comprises the transcriptional termination and polyadenylation sites as well as the SV40 origin of replication and the early and late promoters. Finally, there is the *Sal*I-*Eco*RI fragment of pML-1 that contains the bacterial origin of replication and the β-lactamase gene.

The TGFβ3-coding sequence in the transcription unit of plasmid pRK5-19RR was derived from the porcine TGFβ3 precursor cDNA (16) and was inserted between the *Eco*RI and *Pst*I sites in the polylinker of pRK5. The *Eco*RI site preceded the cDNA sequence starting at nucleotide 255 (16) up-stream from the presumed initiator codon, while the *Xho*I site was at position 1581 in the 3′ untranslated sequence. The more upstream sequences in the 5′ untranslated region, including three ATG triplets, were deleted by site-directed mutagenesis of single stranded DNA (41) subcloned into an M13 derivative. Using the same methodology for site-directed mutagenesis, two codons in the mature TGFβ3-coding sequence were converted into codons for the corresponding human TGFβ3 amino acids, thus changing the derived mature porcine TGFβ3 polypeptide into human TGFβ3. Thus, TGG encoding Ser at position 358 in the precursor (16) was replaced by ACC coding for Thr, and GCC for Ala 394 was converted into CCA for the corresponding human Pro. All site-directed mutagenesis modifications were verified by nucleotide sequence analysis.

The plasmid pRK5-β3-7 contained the coding sequence for the complete human TGFβ3 precursor under the transcriptional control of the CMV-promoter. The cDNA segment was a *Stu*I-*Stu*I fragment containing the cDNA sequence from nucleotide 214 to 1585 (16) and was inserted into the *Sma*I site of the pRK5 polylinker.

The plasmid pSV-dhfr contained a dihydrofolate reductase cDNA under transcriptional control of the SV40 early promoter, similarly to plasmid pFD11 (42).

Cell Culture and Transfections

CHO cells deficient in the synthesis of dhfr (43) were propagated in complete medium, *i.e.* Ham's F-12 and Dulbecco's Modified Eagle's Medium (1:1) supplemented with 10% fetal bovine serum. Cells were transfected with 10 μg TGFβ3 expression plasmid DNA and 200 ng pSV-dhfr DNA as a calcium phosphate coprecipitate (44). Cells were incubated for 3 h, and the medium was removed and replaced by 2 ml PBS containing 20% glycerol for 30 sec at room temperature. The cells were rinsed twice with PBS and incubated in complete medium. After reaching confluence the transfected cells were split 1:7, selected in complete medium lacking glycine, hypoxanthine, and thymidine, and supplemented with 5% dialyzed fetal bovine serum. Individual clones were picked after about 3 weeks. Quantitation of TGFβ in the medium was performed by culturing the confluent cells in the selective medium lacking the bovine serum for 48 h. The medium was then acidified and submitted to a TGFβ bioassay and/or radioreceptor assay.

Amplification of the integrated plasmid sequence was achieved by culturing the cells transfected with plasmid pRK5-19RR in gradually increasing levels of methotrexate. Confluent cultures of each of the three transfected cell clones with the highest bioactivity were split 1:10 and seeded in selective medium containing varying concentrations of methotrexate up to a concentration of 400 nM. Eight clones per transfectant were picked from the 400-nM methotrexate cultures and grown to confluence, and the conditioned medium was assayed as before. Of the 24 clones, the two with the highest bioactivity were selected for further amplification at methotrexate concentrations between 800–1600 nM. From this second round of amplification, the clone with the highest TGFβ3 expression was chosen for purification.

Purification of Recombinant TGFβ3

CHO transfected with pRK5-19RR and resistant to 1600 nM methotrexate were grown to confluence in roller bottles containing 250 ml selective medium with 100 mM HEPES, pH 7.2. The cultures were rinsed with PBS and incubated for 2 days in serum-free medium consisting of Ham's F-12-low glucose Dulbecco's Modified Eagle's Medium (50:50) and 100 mM HEPES, pH 7.2 (100 ml/roller bottle). The purification scheme of the recombinant TGFβ3 was developed on the basis of the methods described by Seyedin *et al.* (45).

Six hundred milliliters of conditioned medium were collected and dialyzed in a Spectrapor D1615-3 dialysis membrane, with a mol wt cut-off range of 12–14 kD, against 16 liters 0.2% acetic acid for days at 4 C. The dialysate was lyophilized to dryness.

The lyophilized conditioned medium was dissolved in 2 ml 6 M urea, 10 mM NaCl, 50 mM NaOAc, and 1 mM *N*-ethylmaleimide, pH 4.8, and centrifuged for 3 min in an Eppendorf centrifuge to remove the insoluble material. The supernatant was then fractionated on a Sephacryl S-200 (Pharmacia, Piscataway, NJ) gel filtration column (60 × 2.5 cm) in the same buffer (flow rate, 31 ml/h). Absorbance at 280 nm was monitored, and fractions of 3 ml were collected. The presence of TGFβ was monitored by the TGFβ bioassay and analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (46).

The fractions containing the peak of TGFβ activity were pooled and further fractionated on a carboxymethyl-cellulose cation exchange column (2.5 × 17 cm; CM-52, Whatman, Clifton, NJ) in the same buffer. The bound proteins were eluted using a 10- to 400-mM NaCl gradient (300 ml), with a flow rate of 30 mM. Again, the presence of TGFβ3 was monitored by TGFβ bioassay and/or SDS-PAGE.

The TGFβ3 pool from the CM-52 column was then loaded onto a C18 reverse phase HPLC column (24 cm × 4.5 mm, Vydac, The Separation Group,Hesparsus, CA). After the urea and salts were washed through, the bound proteins were separated in a 0–60% acetonitrile gradient in 0.1% trifluoroa-

cetic acid over 30 min. The purity of TGFβ3 was assessed by SDS-PAGE (46).

The quantitation of the purified TGFβ3 and determination of amino acid composition and of the N-terminal amino acid sequence were carried out as described previously (47).

TGFβ Bioassay

Six serial dilutions of the column fractions were prepared for each sample in 96-well plates. To each well were added 1×10^4 mink lung epithelial cells (CCL-64) in a final volume of 0.15 ml Minimum Essential Medium-5% fetal bovine serum. After 18–24 h at 37 C, 20 μl 50 μCi/ml [^{3}H]thymidine were added to each well. The plates were then incubated for an additional 4 h, after which time the cells were harvested. The unincorporated radioactivity was removed by extensive washing over glass filters. The incorporated radioactivity retained on the glass filters was then measured. A standard dilution curve using recombinant human TGFβ1 was prepared with each assay, and the bioactivity of each sample was calculated using dilutions that resulted in inhibition close to 50%. TGFβ1 and -β2 have been reported to be equipotent in this assay (26).

TGFβ Radioreceptor Assays

Two radioreceptor assays were used. The assay using A549 cells was used to assess the expression levels of the transfected cells and to follow the purification, while the other using AKR-2B fibroblasts was used to obtain the data shown in Fig. 4.

A549 cells were incubated overnight in Dulbecco's Modified Eagle's Medium-5% fetal bovine serrum in 24-well plates (2×10^5 cells/well), then washed twice with assay diluent [Ham's F-12-Dulbecco's Modified Eagle's Medium (50:50), 0.1% BSA, 25mMHEPES (pH 7.2), and 42.5 mM NaCl]. Cell monolayers were then incubated for 2 h at ambient temperature with dilutions of the TGFβ-containing medium or the human TGFβ1 standard to which ^{125}I-labeled recombinant human TGFβ1 had been added (2×10^5 cpm in 0.2 ml assay diluent). Each well was washed four times with cold PBS containing 0.1% BSA. Well contents were solubilized with 0.75 ml 10% glycerol, 10% Triton X-100, and 25 mM HEPES (30 min at 37 C) and counted by liquid scintillation. TGFβ activity was calculated from the standard curve, using a nonlinear curve-fitting program, and was expressed as TGFβ1 equivalents.

The assay using AKR-2B cells was performed essentially as described previously (48). AKR-2B cells were plated in six-well plastic culture dishes at a density of 2×10^5 cells/well in McCoy's 5A medium with 5% fetal bovine serum. Binding studies were performed 24 h later when the cells reached confluence. The cells were washed three times with PBS containing 0.1% BSA, then were incubated for 1 h at room temperature with constant rocking in 1 ml binding buffer [120 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM HEPES (pH 7.2), and 2 mg/ml BSA]/well. The binding buffer was then replaced with binding buffer containing the appropriate dilution of the conditioned medium and ^{125}I-labeled porcine TGFβ1 (2×10^5 cpm). The cells were incubated for an additional 2 h at room temperature with constant rocking. After binding, the cells were washed three to five times in PBS containing 0.1% BSA, followed by 1 ml/well PBS with 1% Triton X-100 to lyse the cells. The cell lysate was collected after 10 min and counted in a liquid scintillation counter.

[^{3}H]Thymidine Uptake Experiments

Mink lung epithelial cells (CCL-64) were plated in 24-well dishes at 4×10^4 cells/well in McCoy's 5A medium supplemented with 5% fetal bovine serum. Twenty-four hours later the cells were treated with TGFβ1, -β2, or -β3. Twenty-three hours after TGFβ treatment the cells were incubated with [^{3}H]thymidine (4 μCi/ml) for 2 h. After this incubation the relative levels of radioactivity incorporated into trichloroacetic acid-insoluble material were determined.

The experiments using human keratinocytes or the mouse keratinocyte cell line BALB/MK were performed in a similar way, but the cells were cultured for 48 h before TGFβ treatment and treated with [^{3}H]thymidine for 24–30 h after treatment with TGFβ. Human keratinocytes were grown in KBM medium (Clonetics, Inc.), supplemented with 0.05 mM calcium, 5 μg/ml insulin, 10 ng/ml EGF, and 0.05% fetal bovine serum. The mouse keratinocytes were cultured in Miminum Essential Medium supplemented with 0.05 mM calcium, 8% dialyzed fetal bovine serum, and 4 ng/ml EGF.

[^{3}H]Thymidine incorporation in AKR-2B mouse fibroblasts was measured as described above for CCL64 cells. The cells were grown for 48 h in McCoy's 5A medium supplemented with 5% bovine serum. The medium was then changed to serum-free medium. Upon quiescence of the cells, *i.e.* 48 h after serum-free medium was added, fresh serum-free medium containing TGFβ1, -β2, or -β3 and supplemented with 500 ng/ml insulin was added to the quiescent cells. The cells were treated with [^{3}H]thymidine 30–48 h after the TGFβ additions, and the incorporated radioactivity was measured as described above.

Growth Inhibition of MCF-7 Cells

MCF-7 cells were seeded at 4.5×10^4 cells/well in 1.5 ml RPMI-5% fetal bovine serum in 24-well plates. The next day, medium was replaced with fresh medium containing TGFβ1, -β2, or -β3. After 3 days, medium and TGFβ were replaced. Two days later, cell monolayers were harvested with trypsin treatment, and the cells were counted using a Coulter counter (Hialeah, FL). Cell counts from wells not exposed to TGFβ were used to calculate the percent inhibition of cell proliferation.

Soft Agar Colony Formation

Soft agar assays were performed using AKR-2B (clone 84A) cells as the indicator cells, as previously described. Cells (1×10^4/dish) were seeded, and the indicated amount of TGFβ1, -β2, or -β3 was added. Colonies were counted after 7–10 days, using a computer-assisted soft agar colony counter.

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