Stimulation of Collagen Synthesis by the Anabolic Steroid Stanozolol

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There is evidence that anabolic steroids, which are derived from testosterone and have markedly less androgenic activity, promote tissue growth and enhance tissue repair; however, the mechanisms involved in their anabolic activities remain unclear. In this report, we measured the effect of the anabolic steroid stanozolol on cell replication and collagen synthesis in cultures of adult human dermal fibroblasts. Stanozolol (0.625–5 μg per ml) had no effect on fibroblast replication and cell viability ($p = 0.764$) but enhanced collagen synthesis ($p < 0.01$) in a dose-dependent manner ($r = 0.907$). Stanozolol also increased (by 2-fold) the mRNA levels of α1(I) and α1(III) procollagen and, to a similar extent, upregulated transforming growth factor-β1 (TGF-β1) mRNA and peptide levels ($p < 0.001$). There was no stimulation of collagen synthesis by testosterone. The stimulatory effects of stanozolol on collagen synthesis were blocked by a TGF-β1 anti-sense oligonucleotide, by antibodies to TGF-β, and in dermal fibroblast cultures derived from TGF-β 1 knockout mice. We conclude that collagen synthesis is increased by the anabolic steroid stanozolol and that, for the most part, this effect is due to TGF-β1. These findings point to a novel mechanism of action of anabolic steroids. ***Key words: fibroblasts/transforming growth factor-β/wound healing.*** ***J Invest Dermatol 111:1193–1197, 1998***

Anabolic steroids are synthetic derivatives of testosterone produced to dissociate testosterone's anabolic and androgenic action (Wilson, 1990). Although the abuse of anabolic steroids for increasing muscle mass and for improving physical performance is well documented, the accepted therapeutic indications of these agents are seemingly unrelated to their anabolic effects; stanozolol is an accepted treatment for angiedema and endometriosis (Wilson, 1990; Helfman and Falanga, 1995). In recent years, however, there has been increasing interest in the anabolic actions of these drugs, with emphasis on such clinical applications as aging (Helfman and Falanga, 1995), wound repair (Browse *et al*, 1977; Falanga *et al*, 1991), and HIV-related wasting myopathy (Berger *et al*, 1993). The potential use of anabolic steroids in wound healing has received particular attention. When administered either preoperatively or postoperatively, anabolic steroids reverse the deleterious effects of corticosteroids on experimental intestinal anastomotic healing (Kim *et al*, 1993) and bone repair (Helfman and Falanga, 1995). Recently, we have shown that stanozolol, a synthetic anabolic steroid with one of the largest anabolic/androgenic ratios (Wilson, 1990), causes dramatic healing of certain types of ischemic ulcerations (Falanga *et al*, 1991; Kirsner *et al*, 1993). There are, however, few investigations focused on the mechanisms of action of stanozolol and other anabolic steroids. An *in vivo* study reported that anabolic steroids enhance tensile strength of wounds by increasing the amount of chondroitin sulfate (Watts *et al*, 1965). Using dermal fibroblast cultures, others have shown that stanozolol stimulates procollagenase production (Wright *et al*, 1989). In this report, we determined the effect of stanozolol on human dermal fibroblast replication and collagen synthesis. The results shown here indicate that stanozolol stimulates collagen synthesis and that, for the most part, this effect is due to the action of transforming growth factor-β1 (TGF-β1).

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MATERIALS AND METHODS

Fibroblast cultures and cell counts Adult human dermal fibroblasts were cultured from the dorsal forearm of a total of seven healthy donors, as previously described (Falanga *et al*, 1987). When so specified, we also used neonatal foreskin fibroblasts in their first two *in vitro* passages. Cultures were established and expanded in T-75 flasks (Costar, Cambridge, MA) with Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (GIBCO, Grand Island, NY) at 37°C, 95% air, and 5% CO_2. For actual experiments, AIM V serum-free media (GIBCO) was used. In all experiments involving collagen measurements, 100 μM ascorbic acid (Sigma, St. Louis, MO) were added. For measurements of cell replication, fibroblasts were seeded at a density of 5000 cells per well into 24 micro well dishes. After seeding, cultures were stimulated at days 1 and 4 with either control media or stanozolol (Research Plus, Bayonne, NJ), dissolved in dimethylsulfoxide (maximal concentration of dimethylsulfoxide of 0.001%). In these and other experiments the same concentration of dimethylsulfoxide was added to control media. Stanozolol was added in concentrations ranging from 0.625 (1.9×10^{-6}M) to 5 μg per ml. Cell numbers were measured with a hemacytometer. Cell viability was determined by color exclusion after mixing equal volumes of cell suspension with trypan blue solution (0.4% in 0.81% NaCl) in the absence of serum.

For the purpose of establishing cultures of TGF-β1 knockout dermal fibroblasts, skin from TGF-β1 knockout mice and control litter mates (Kulkarni *et al*, 1993) was provided by one of us (A.B. Roberts). Dermal fibroblasts cultured from mouse skin were maintained in DMEM plus 10% fetal bovine serum until actual experiments, at which time cultures were washed extensively with DMEM, and serum-free medium (AIM-V) was added.

Measurements of collagenous protein Fibroblasts were seeded into 24 micro well culture dishes at a density of 50,000 cells per well and at confluence

(2–3 d later) were stimulated with stanozolol (0.625–5 μg per ml). Replicate cultures were stimulated with human TGF-β1 (R&D Systems, Minneapolis, MN). At this time, [^{3}H]proline (99 Ci per mmol; Amersham, Arlington Heights, IL) was added to each well at a concentration of 20 μCi per well. The total volume in each well was 0.4 ml. After 48 h, [^{3}H]proline incorporation into pepsin-resistant, salt precipitable extracellular collagen was determined as previously described (Webster and Harvey, 1979; Takagi *et al*, 1995). Results were expressed as cpm of [^{3}H]collagen per cell number, as determined with a hemacytometer.

TGF-β1 peptide levels These were measured, as recently described (Hasan *et al*, 1997), with an enzyme-linked immunosorbent assay kit from R&D Systems and following the manufacturer's protocol. Six-well plates were seeded with 2.5×10^5 per well of fibroblasts in 0.2 ml of DMEM supplemented with 10% fetal bovine serum. The medium was then changed to DMEM without serum supplemented with 200 μg crystalline bovine serum albumin (Sigma) per ml, with four changes of medium over 24 h to remove serum and excess TGF-β1. Cultures were then incubated for an additional 24 h in DMEM without serum. At the end of this 24 h period, the supernatant from each culture (conditioned media) was collected, centrifuged at 10,000 r.p.m. for 10 min, and stored at –70°C. Prior to freezing, each sample received 2 μg aprotinin, leupeptin, and pepstatin A per ml, plus 120 μg phenylmethylsulfonyl fluoride (all from Sigma) per ml. For measurements, the samples were thawed and TGF-β in samples was activated by adding 0.2 ml of 1 N HCl to each 1.0 ml of conditioned media to give a final concentration of 0.167 N HCl. After 10 min at room temperature, each acidified sample was neutralized with 0.2 ml of 1.2 N NaOH/0.5 M HEPES. Thereafter, 0.2 ml of each conditioned media sample was added per well into a 96 well plate, the bottom of which was coated with recombinant TGF-β soluble Type II receptor. A 1:1 dilution series for TGF-β1 standards was prepared starting at 2000 pg per ml (2000, 1000, 500, 250, 125, 62.5, 31.2, and 0 pg per ml). Standards were assayed in duplicates, and samples were measured in quadruplicate. Each sample and standard were incubated in each receptor-coated well for 3 h at room temperature. The supernatant from each well was then aspirated and discarded and each well was washed three times with a wash buffer (buffered surfactant, as per the manufacturer). To each well were then added 0.2 ml of polyclonal antibody against TGF-β1 conjugated to horseradish peroxidase. After 1.5 h, the supernatant was removed and each well was washed three times with wash buffer. A hydrogen peroxide-chromogen mixture (0.2 ml) was then added to each well for 20 min at room temperature. The reaction was stopped by adding 0.05 ml of 2N sulfuric acid, and optical density was measured at 450 nm.

RNA extraction and northern analysis For all experiments, fibroblasts were grown to near confluence, to a density ranging from 0.75 to 1.0×10^6 cells per T-75 flask. Total cellular RNA from cells was isolated by extraction in guanidium isothiocyanate using the method of Chomczynski and Sacchi (1987). It was then separated for northern blot analysis on 1% agarose gels containing 5% formaldehyde and transferred to a nylon membrane (Schleicher and Schuell, Keene, NH) in a gradient of 20×to 10×sodium citrate/chloride buffer. The following cDNA probes were used: a 1.5 kb EcoR1 fragment of cDNA from the original clone Hf677 for the α1(I) procollagen chain (Chu *et al*, 1982); a 1.4 kb Pst1 fragment of the cDNA clone pH III 33 coding region for the α1(III) procollagen chain (Miskulin *et al*, 1986); and a 1.1 kb EcoR1 fragment of the TGF-β1 cDNA (Derynck *et al*, 1985). Plasmids for these cDNA and for GAPDH cDNA (used as a housekeeping gene) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). Probes were labeled with ^{32}P by random priming and used for northern blot analysis as previously described (Falanga *et al*, 1993). For RNA electrophoresis, 10 μg of total RNA was loaded per lane, as measured by absorbance at 260 nm. Confirmation of uniformity of RNA loading was obtained by staining the nylon blots with methylene blue (Falanga *et al*, 1993). Northern hybridization was performed at 42°C in a solution containing 50% formamide, 6×sodium citrate/chloride buffer, 5×Denhardt's reagent, 0.5% sodium dodecyl sulfate, and the labeled cDNA probe (2×10^{-8} cpm per mg). The blots were washed at room temperature one time in 1×sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate for 20 min, followed by three washes at 68°C in 0.2×sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate for 20 min each. Autoradiography was generally carried out overnight at –70°C.

Anti-sense oligonucleotides We used 0.1–10 μM of a TGF-β1 19′mer anti-sense oligonucleotide (5′ gAg ggC ggC ATg ggg gAg g 3′), which overlaps the promoter and transcriptional start site of the TGF-β1 gene. This same sequence, which is specific for the TGF-β1 isoform, has been used successfully to block TGF-β1 transcription *in vitro* (Murata *et al*, 1997) and *in vivo* (Brunet *et al*, 1995). Confluent fibroblast cultures were either left untreated in AIM-V, or treated with stanozolol alone or in combination with three different concentrations of the anti-sense oligonucleotide. Sense oligonucleotide served as an additional control. RNA isolation and measurements of procollagen mRNA levels were made 24 h later.

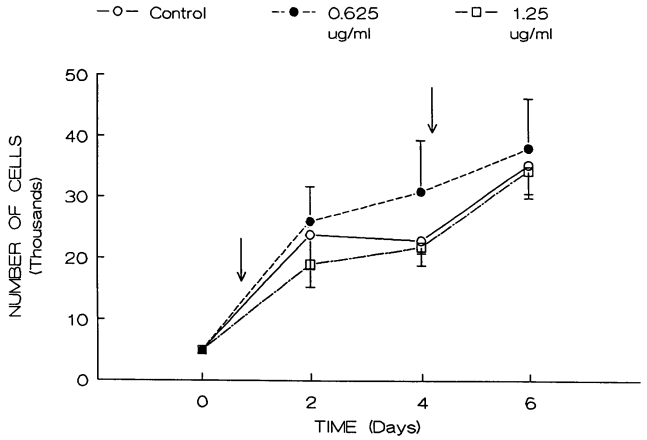

Figure 1. Stanozolol does not stimulate fibroblast replication. Stanozolol was added to cultures on days 1 and 4 after seeding 5000 adult dermal fibroblasts per well. The results represent the mean ± SD from quadruplicate wells. The *arrows* show the time points at which stanozolol was added to the cultures.

TGF-β1 antibodies We used a polyclonal neutralizing antibody to TGF-β (1D11 antibody, Genzyme, Cambridge, MA), which has been previously used to block the activity of this cytokine *in vitro* (Falanga *et al*, 1993). This antibody is not specific for the TGF-β1 isoform, as it also blocks the effect of TGF-β2. Fibroblast cultures were washed extensively with 0.1% bovine serum albumin in DMEM to remove excess serum and TGF-β peptides. They were then either left untreated in AIM-V or treated for 24 h with stanozolol alone or in combination with the antibody.

Statistical analysis Data were entered in a computerized statistical analysis program (InStat; GraphPAD Software, San Diego, CA). The Student's t test and one-way analysis of variance test were used for parametric results, whereas linear regression analysis was employed to determine correlation coefficients (r). Statistical significance was defined as a p value of 0.05 or less.

RESULTS

Stanozolol has no effect on fibroblast proliferation We tested different concentrations of stanozolol, at different seeding densities, and with varying dosing schedules. We found no effect of stanozolol on cell replication, and **Fig 1** shows a representative experiment. Cell viability was not affected by stanozolol, as determined by trypan blue exclusion in more than 95% of the cells.

Stanozolol increases collagen synthesis and α1(I) procollagen mRNA levels We determined the effect of stanozolol on [^{3}H]proline incorporation into pepsin-resistant, salt precipitable extracellular collagen, using a previously reported method (Takagi *et al*, 1995). As seen in the representative experiment shown in **Fig 2**, stanozolol increased collagen synthesis by 35% ($p < 0.01$). In the same experiment, higher concentrations of stanozolol (1.25 and 2.5 μg per ml) were as effective in stimulating collagen synthesis as TGF-β1 (5 ng per ml), which was used here as an additional (positive) control. These results indicate that stanozolol increases overall collagenous protein synthesis. Total protein released in the culture medium was not increased by stanozolol ($p > 0.05$; data not shown). We next determined the effect of stanozolol on mRNA levels of type I and type III procollagen. For these experiments, fibroblasts were grown to confluence in DMEM plus 10% fetal bovine serum. Cultures were then washed twice with phosphate-buffered saline and the media were changed to serum-free AIM-V plus different concentrations of stanozolol for 24 h. These experiments were done four times, and with similar results. A representative experiment is shown in **Fig 3**, where it can be seen that stanozolol caused a dose-dependent increase in the mRNA levels of α1(I) and α1(III) procollagen, and TGF-β1. These stimulatory effects of stanozolol on procollagen are most likely due to its anabolic rather than androgenic properties. Thus, as shown in **Fig 4**, similar molar concentrations of testosterone (0.625–5 μg per ml) failed to stimulate

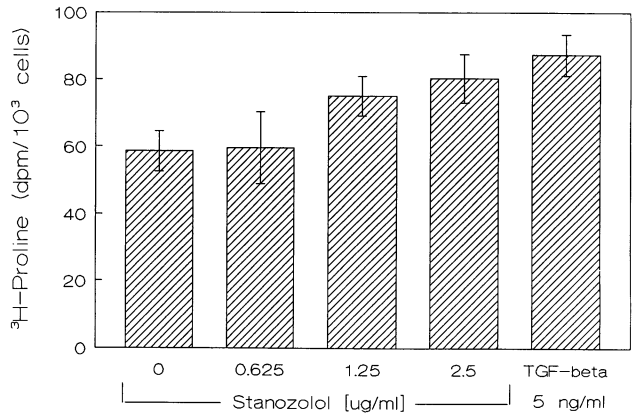

Figure 2. Increased collagenous protein in response to stanozolol. Adult dermal fibroblasts were seeded at 50,000 cells per 2.1 cm^2 well and, after 2 d (at confluence), were pulsed for 24 h with 20 uCi [^{3}H]proline. Results represent the mean ± SD from quadruplicate wells.

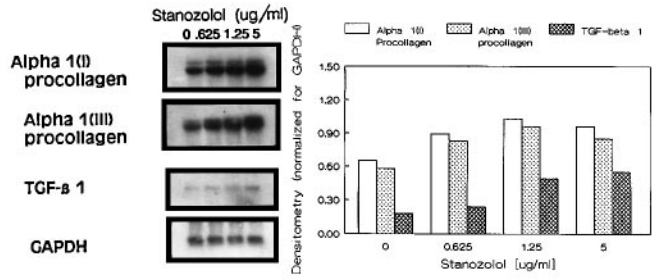

Figure 3. Stanozolol increases mRNA levels of α1(I) and α1(III) procollagen and TGF-β1. Total cellular RNA was isolated from duplicate confluent cultures of adult dermal fibroblasts after 48 h of exposure to stanozolol. The figure shows the northern analysis and densitometric determination of each band.

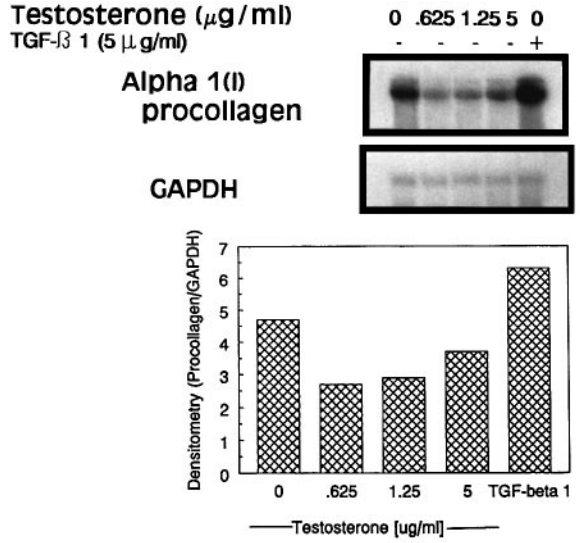

Figure 4. Testosterone fails to increase α1(I) procollagen mRNA levels. Total cellular RNA was isolated from duplicate confluent cultures of adult dermal fibroblasts after 48 h of exposure to testosterone. The figure shows the northern analysis and densitometric determination of each band.

and actually decreased α1(I) procollagen mRNA levels when compared with baseline measurements.

The stimulatory effects of stanozolol are due to TGF-β1 We hypothesized that the action of stanozolol may be mediated by TGF-β1. TGF-β1 is a potent stimulus for collagen synthesis (Roberts *et al*, 1986; Varga *et al*, 1987) and, as our data indicated, its mRNA levels

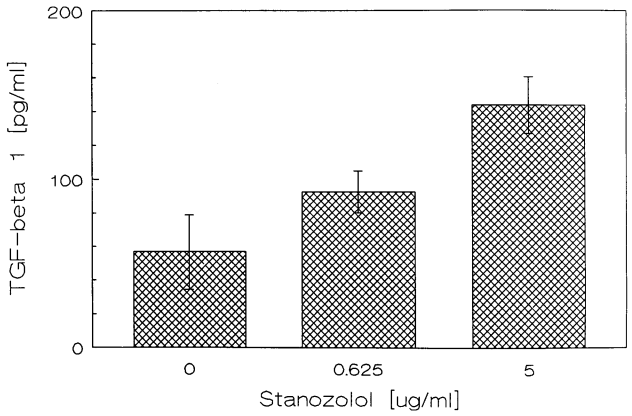

Figure 5. Stanozolol increases TGF-β1 peptide levels. TGF-β1 peptide levels were measured by enzyme-linked immunosorbent assay in serum-free media conditioned for 24 h by cultures of adult dermal fibroblasts in the presence or absence of stanozolol. The results are the mean ± SD from quadruplicate wells.

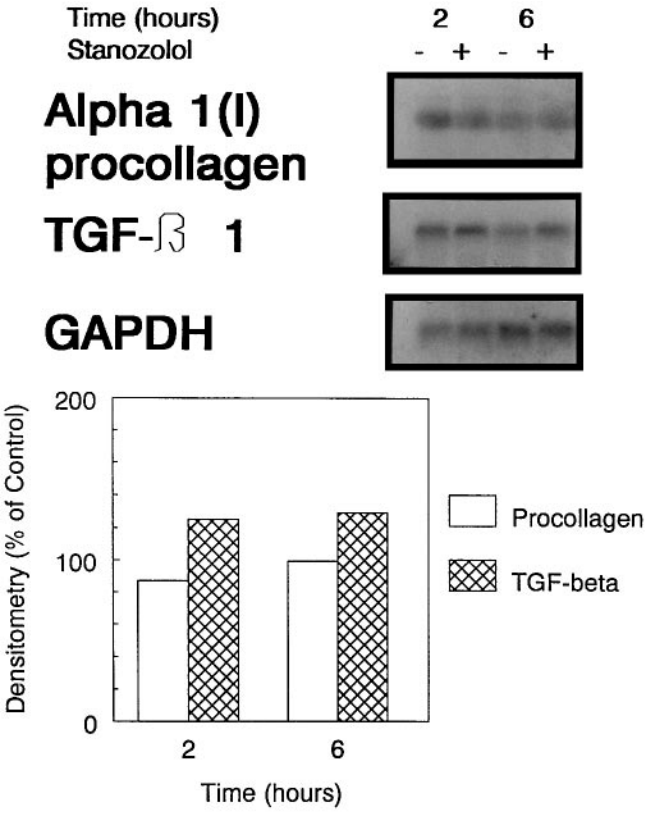

Figure 6. Stanozolol causes an early increase in TGF-β1 mRNA levels. Total cellular RNA was isolated from duplicate confluent cultures of adult dermal fibroblasts after 2 or 6 h of exposure to stanozolol (2.5 μg per ml). The figure shows the northern analysis and densitometric determination of each band. The data in the graph have been normalized for GAPDH.

are increased by stanozolol (**Fig 3**). In the next series of experiments, we measured TGF-β1 peptide synthesis in response to stanozolol. TGF-β1 levels were measured by enzyme-linked immunosorbent assay (R&D Systems), using the type II TGF-β receptor as a substrate and an antibody specific for TGF-β1 for detection. As shown in **Fig 5**, stanozolol increased TGF-β1 peptide levels by as much as 200% ($p < 0.001$). Therefore, stanozolol increases both collagen and TGF-β1 synthesis. In fact, mRNA levels of TGF-β1 are increased as early as 2 h after exposure of fibroblasts to stanozolol, and before procollagen mRNA levels are increased (**Fig 6**). We next asked whether TGF-β1 is a critical factor in the stimulation of collagen synthesis by stanozolol.

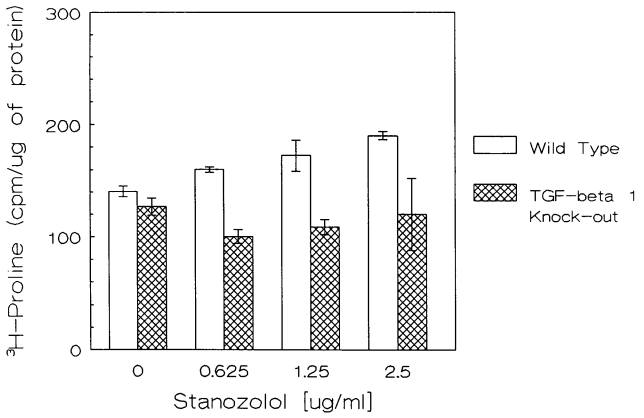

Figure 7. TGF-β1 knockout fibroblasts fail to respond to stanozolol. Dermal fibroblasts from TGF-β1 knockout mice and control litter mates were exposed to stanozolol for 24 h before measuring collagenous protein synthesis. The results are the mean ± SD from quadruplicate wells.

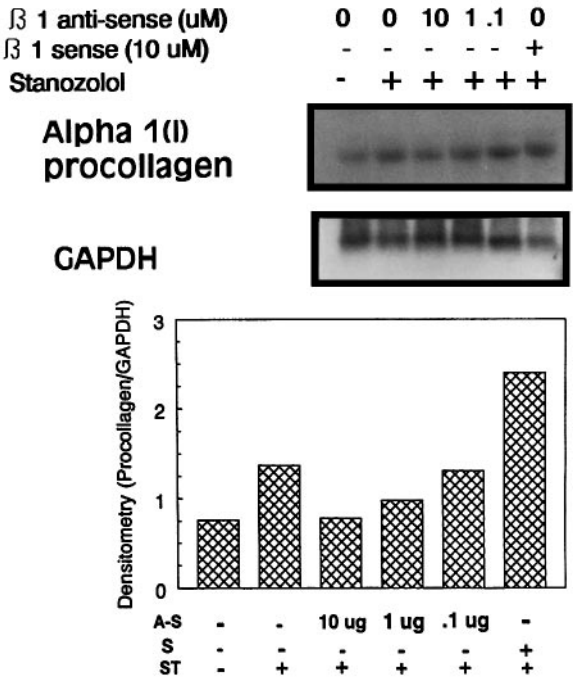

Figure 8. TGF-β1 anti-sense oligonucleotide blocks the stimulatory effect of stanozolol on α1(I) procollagen mRNA. Human dermal fibroblasts were first exposed for 24 h to increasing concentrations of a 19′mer TGF-β1 anti-sense oligonucleotide in serum-free medium. Cultures were then either left untreated or exposed for an additional 24 h to stanozolol or to sense oligonucleotide, which served as an additional control. The graph shows the densitometric analysis of the northern blot for α1(I) procollagen, and the data are normalized for GAPDH. A-S, anti-sense oligonucleotide; S, sense oligonucleotide; ST, stanozolol.

We approached this question in three ways. First, we tested the effect of stanozolol in an environment devoid of TGF-β1. For this purpose, we derived dermal fibroblast cultures from skin samples of TGF-β1 knockout mice and control litter mates (Kulkarni *et al*, 1993). We then used these cultures to measure the effect of stanozolol in the absence of TGF-β1. As shown in **Fig 7**, stanozolol increased overall collagen synthesis in fibroblast cultures from control litter mates ($p < 0.002$) but not in fibroblasts from TGF-β1 knockout mice, indicating that the stimulatory action of stanozolol on collagen synthesis is, in large part, due to TGF-β1. We next determined whether a TGF-β1 anti-sense oligonucleotide would block the stimulatory effect of stanozolol. We used a 19′mer oligonucleotide that has been shown previously to block TGF-β1 activity (Brunet *et al*, 1995; Murata *et al*, 1997). As shown in **Fig 8**, the TGF-β1 anti-sense oligonucleotide blocked the

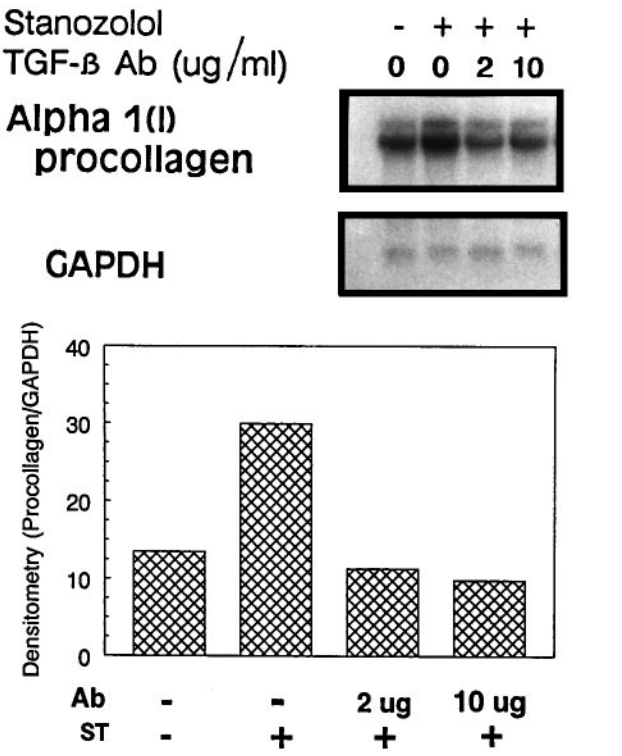

Figure 9. TGF-β antibodies block the stimulatory effect of stanozolol on α1(I) procollagen mRNA. Confluent cultures of human dermal fibroblasts were extensively washed to remove excess TGF-β peptides and then either placed in serum-free media (AIM-V) alone or with stanozolol plus neutralizing antibody to TGF-β for 24 h. The graph shows the densitometric analysis of the α1(I) procollagen bands normalized for GAPDH. ST, stanozolol; Ab, antibodies.

stimulation of α1(I) procollagen mRNA levels by stanozolol in a dose-dependent manner; sense oligonucleotide had no effect. Similarly, as observed in **Fig 9**, the stimulation of procollagen mRNA levels by stanozolol was blocked by antibodies to TGF-β. Taken together, these results strongly point to TGF-β1 as a critical cytokine involved in the observed stimulation of collagen synthesis by stanozolol.

DISCUSSION

We report that the anabolic steroid stanozolol stimulated overall collagen synthesis and increased the mRNA levels of α1(I) and α1(III) procollagen. These stimulatory effects of stanozolol on collagen synthesis were not observed with testosterone and were accompanied by increased synthesis of TGF-β1. The mRNA levels of TGF-β1 were increased as early as 2 h after exposure of fibroblasts to stanozolol, and before any increase in procollagen mRNA. We found that stanozolol failed to stimulate collagen synthesis in TGF-β1 knockout fibroblasts and after the addition of a TGF-β1 anti-sense oligonucleotide and antibodies to TGF-β. Taken together, these findings strongly suggest that the stimulation of collagen synthesis by stanozolol is due, in large part, to the action of TGF-β1.

Although the increase (35%) in collagenous protein observed in this study may appear modest at first, it should be noted that the experiments were done in a defined serum-free media (AIM-V) without added growth factors, so as to avoid binding of stanozolol to steroid binding proteins. Moreover, the amount of collagen synthesis observed in response to stanozolol was the same as that measured after the addition of TGF-β1 and, importantly, the stimulatory actions of stanozolol appear to be specific, in that the parent compound testosterone actually decreased collagen synthesis.

Evidence linking stanozolol or other anabolic steroids to increased extracellular matrix formation has been limited thus far. In one report, stanozolol was found to enhance procollagenase production by dermal fibroblasts but not in synovial fibroblast cultures (Wright *et al*, 1989);

however, collagenous protein was not measured in that study. Stanozolol has also been shown to stimulate prostaglandin E2 (PGE2) synthesis and to inhibit fibroblast growth factor-induced DNA synthesis in human skin fibroblasts (Ellis *et al*, 1994). In animal studies, stanozolol has been reported to increase muscle protein synthesis without a direct effect on protein degradation (Gribbin and Flavell Matts, 1976; Helfman and Falanga, 1995). *In vivo* studies in humans have not specifically addressed the effects of anabolic steroids on extracellular matrix formation, but do point to overall anabolic activity. For example, a short-term study of 16 patients, eight of whom received 10 mg of stanozolol orally each day for 14–21 d, showed an increase in the bulk of type I (oxidative) fibers in response to stanozolol (Hosegood and Franks, 1988). Other reports suggest that stanozolol may be effective in the treatment of osteoporosis, in improving nitrogen balance in wasting diseases such as muscular dystrophy, and in postoperative trauma (Glueck *et al*, 1995). Stanozolol has also been shown to cause dramatic healing of painful cutaneous ulcerations due to cryofibrinogenemia (Falanga *et al*, 1991; Kirsner *et al*, 1993); however, it is unclear whether the beneficial effects of stanozolol in these dermal ischemic wounds is due to direct stimulation of tissue repair or to the fibrinolytic action of stanozolol (Browse *et al*, 1977).

TGF-β1 is an established stimulus for the formation of extracellular matrix both *in vitro* (Roberts *et al*, 1986; Varga *et al*, 1987) and *in vivo* (Roberts *et al*, 1986; Mustoe *et al*, 1987). The results shown here point to TGF-β1 being critically involved in the stimulatory action of anabolic steroids on collagen synthesis. This observation is of interest when one considers the possibility of using anabolic steroids to offset the deleterious effects of corticosteroids on wound healing. For example, in one study of experimental cutaneous wounds in rats, it was shown that the systemic administration of TGF-β1 reversed the inhibitory effects of corticosteroids on healing (Beck *et al*, 1993). In another report of anastomotic healing of intestinal wounds, stanozolol reversed the inhibition of healing caused by corticosteroids (Kim *et al*, 1993). Therefore, it is plausible that TGF-β1 plays an important role in the way anabolic steroids work, or at least in the way they oppose some of the effects of corticosteroids.

In conclusion, we provide evidence that stanozolol stimulates collagen synthesis. If these effects are mediated through TGF-β1, as our results indicate, we should start thinking of anabolic steroids as acting pharmacologically to increase the synthesis of potent growth factors and cytokines.

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