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Prevention of Cell Death Induced by Tumor Necrosis Factor α in LNCaP Cells by Overexpression of Sulfated Glycoprotein-2 (Clusterin)[1]

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ABSTRACT

Sulfated glycoprotein-2 (SGP-2) expression has been associated with programmed cell death in the prostate, but its exact role remains unclear. The present study was carried out in an attempt to establish the function of SGP-2 in programmed cell death using tumor necrosis factor (TNF)α-induced cytotoxicity in LNCaP cells as the model system. LNCaP is an androgen-sensitive, human prostatic cancer cell line that responds to TNF in culture by undergoing programmed cell death, as determined by the loss of cell number, failure to exclude trypan blue, detection of DNA fragmentation, and increased release of previously incorporated [^{3}H]thymidine. Immunocytochemical staining for SGP-2 was weak but evident in LNCaP cells. Following treatment with TNFα, there was a time-dependent increase in SGP-2 staining, the intensity of which peaked at 2 h and declined thereafter. SGP-2 staining in LNCaP cells was undetectable prior to the onset of DNA fragmentation at 6 h of TNF treatment. This observation indicated that TNF-induced cell death in LNCaP cells was characterized by an initial transient elevation of SGP-2, followed by a period of SGP-2 depletion that preceded cell death. Transfection of LNCaP with a 21-base oligonucleotide antisense to SGP-2 resulted in a significant increase in cell death that was sequence specific and was accompanied by a reduction in SGP-2 biosynthesis. These findings supported the concept that SGP-2 depletion, rather than its expression, was associated with cell death. Finally, stable transfection and subsequent overexpression of SGP-2 in LNCaP cells resulted in resistance to the cytotoxic effect of TNF. These results have provided evidence to indicate that SGP-2 plays a role in the protection of TNF-induced cell death in LNCaP cells.

INTRODUCTION

SGP-2,[3] also known as clusterin, is a ubiquitous protein with a diverse range of proposed activities (1). In the rat prostate, SGP-2 expression is associated with castration-induced programmed cell death (2–5) and is also known as the testosterone repressed message (TRPM-2; Ref. 4). During castration-induced prostatic regression in rats, there is an immediate increase in SGP-2 message as well as protein levels in the prostate. These levels reach a peak at 3–4 days postcastration and coincide with the onset of massive cell death (6–11). This elevated level of SGP-2 is associated with the epithelial cells and seems to be transient, because it declines significantly thereafter (11). These findings, along with findings in other organ systems, (12, 13), have led to the concept that SGP-2 is a marker for cell death. In fact, many investigators have used the expression of SGP-2 in prostatic cells and other cells as an indication of cell death (9, 14, 15). However, other studies have provided conflicting information, suggesting that SGP-2 expression is not related to cell death. For example, Sertoli cells in the rat testis are rich sources of SGP-2 under normal physiological conditions, and its expression has not been linked with Sertoli cell death (16–19). Results of our recent studies of the rat prostatic ductal system (7, 10) have also indicated that not all prostatic epithelial cells that express SGP-2 are destined for cell death. These observations have left the possibility of SGP-2 being associated with prostatic cell death questionable; much less certain is the role of SGP-2 in cell death.

In an attempt to identify the exact role of SGP-2 in prostatic cell death, an *in vitro* tissue culture system using LNCaP cells has been adopted (20). LNCaP is an androgen-sensitive human prostatic cancer cell line derived from a supraclavicular lymph node of a patient with metastatic Stage D2 prostate cancer (21, 22). This line is exquisitely sensitive to the cytotoxic effect of TNFα (20). The present study was, therefore, carried out to investigate the role of SGP-2 in TNF-induced cell death in LNCaP and to provide an explanation toward biological events of castration-induced cell death in the rat prostate as it relates to SGP-2 expression.

MATERIALS AND METHODS

Cells and Culture Conditions. Parental LNCaP cells were purchased from the American Type Culture Collection (Rockville, MD). All cells were maintained in RPMI 1640 containing 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 μg/ml). Culture media for SGP-2-transfected LNCaP clones was supplemented with 200 μg/ml G418 (Life Technologies, Gaithersburg, MD). In experiments using TNFα or phosphorothioate oligodeoxynucleotides, control and test media consisted of RPMI 1640 supplemented with ITS Plus (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenous acid, 1.25 mg/ml BSA, and 5 μg/ml linoleic acid; Collaborative Research, Inc., Bedford, MA) supplemented with or without 10 ng/ml recombinant TNFα (specific activity, 6.27 × 10^7 units/mg; Collaborative Research, Inc., Bedford, MA) or oligonucleotides, respectively.

Synthesis of Antisense Oligonucleotides. Twenty-one mer phosphorothioate oligodeoxynucleotides sense and antisense to various regions of human SGP-2 message (23) were synthesized (Synthcell Corp., Rockville, MD). The sequences were: AS 1-21, 5′ ATT GTC TGA GAC CGT CTG GTC 3′; AS 22-42, 5′ ATT GGA CAT TTC CTG GAG CTC 3′; AS 43-64, 5′ ATT GAC GTA CTT ACT TCC CTG 3′; and AS 65-86; 5′ GAC AGC ATT TTG AAT TTC CTT 3′. Oligonucleotide AS 1-21 consisted of bases antisense to a region of SGP-2 mRNA extending from the start site of the message through the next 21 bases. The subsequent antisense oligomers were constructed sequentially along the bases and were designated as AS 22-42, AS 43-64, and AS 65-86. The corresponding sense oligodeoxynucleotides were also synthesized as controls. An antisense oligodeoxynucleotide to the first 21 bases of albumin mRNA (ALB) was synthesized as an additional control (24).

Cell Counting, Trypan Blue Dye Exclusion, and Cytotoxicity Assay. For determination of cell number, cells in 24-well plates were detached by 0.5 ml trypsin-EDTA solution (Hazelton Biologics, Lenexna, KS). The detached cell solution was transferred to a counting vessel containing 9.5 ml isotonic solution (Isoton II; Coulter Corp., Hialeah, FL) and counted using a Coulter counter (Coulter). Trypan blue dye exclusion test was performed to determine cell viability.

A cytotoxic assay was also conducted to assess the extent of cell death. Cells were prelabeled with [^{3}H]thymidine (5 μCi/ml; Amersham Corp., Arlington

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[3] The abbreviations used are: SGP-2, sulfated glycoprotein-2; TNF, tumor necrosis factor.

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Heights, IL) for 48 h. At the indicated times after TNF or oligonucleotide treatment, the amount of radioactivity released into the culture well was calculated as (25):

$$\% \text{ of radioactivity released} = \frac{\text{Total dpm} - \text{dpm remaining in cells}}{\text{Total dpm}} \times 100$$

where *Total dpm* represented the amount of radioactivity present in cells plus the amount released into the culture well.

DNA Fragmentation Assay. Analysis of the extent of DNA fragmentation was conducted according to the procedure described by Kyprianou and Isaacs (8). LNCaP cells were plated onto T-150 flasks and treated with TNFα for various time periods. Cells from two flasks were pooled for the isolation of DNA. A commercial kit (A.S.A.P.; Boehringer-Mannheim, Indianapolis, IN) was used to isolate DNA. Briefly, harvested cells, adherent and floating, were lysed with lysis buffer and treated sequentially with RNase at 37°C and proteinase K at 55°C for 30 min each. The DNA preparation was then applied onto an affinity column, washed, and eluted. The eluted DNA was precipitated with isopropanol, washed with ethanol, and air dried. DNA samples (12–15 μg) were separated by electrophoresis in a 1.8% agarose gel using a Bio-Rad horizontal gel apparatus with 88 mM Tris, 88 nM boric acid, and 2 mM EDTA as the running buffer. A DNA molecular weight standard, ranging from 154–2176 bp (Boehringer-Mannheim) was used as a molecular weight marker. Ethidium bromide (0.2 μg/ml) was included in the running buffer to visualize DNA bands. The migration pattern of the DNA was visualized by a short wave UV transilluminator (Fotodyne, Hartland, WI). Gels were photographed with a Polaroid camera system (model MP-4 land camera with 545 filmholder) using Polaroid high speed film (type 57, 4 x 5 instant sheet film; Polaroid Corp., Cambridge, MA).

Recombinant SGP-2 Expression Vector and Transfection of LNCaP Cells. A 1309-bp cDNA fragment containing the entire open reading frame of rat SGP-2 (bases 55–1413; Ref. 17) was amplified by PCR performed on reverse transcribed mRNA extracted from rat ventral prostate glands as described previously (26). Briefly, prostate mRNA was copied to cDNA in a reaction buffer containing 50 mM Tris (pH 8.3), 40 mM KCl, 10 mM DTT, 6 mM $MgCl_2$, 1 mM each of dATP, dCTP, dTTP, dGTP, and 0.5 μg oligo(dT) using 200 units Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) in a 50-μl reaction for 90 min at 37°C. A 10-μl aliquot of the reverse transcriptase reaction was used for PCR in a reaction containing 20 pM of each 25-base oligomer primer, 20 mM pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, dCTP, dTTP, dGTP, and 0.5 unit Taq polymerase (Life Technologies). Amplification was accomplished by thermocycling for 30 cycles with annealing temperature at 65°C (1.25 min), extension temperature of 72°C (1.5 min) and denaturation temperature of 95°C (1 min). The reaction product was electrophoresed through 1.5% agarose gel, and the 1309-bp fragment was cut from the gel and purified using a Quiagen gel extraction kit (Qiagen, Inc., Chatsworth, CA). The purified fragment was then digested with *Hin*dIII and *Xho*I and ligated into the pBK-CMV expression vector (Stratagene Cloning Systems, Inc., La Jolla, CA; Ref. 27).

LNCaP cells grown in 10-cm^2 dishes were transfected with the pBK-CMV/SGP-2 expression vector (30 μg/dish) or with a control, neomycin-resistance alone, expression vector (pBK-CMV) at 30 μg/dish using Lipofectin (5 μg/dish) in OPTI-MEM medium according to the manufacturer's directions (Life Technologies). Culture medium was replaced 4 h later with RPMI 1640 supplemented with 10% FBS and, at 24 h, was replaced with the above medium supplemented with 600 μg/ml G418 (Life Technologies). Colonies were selected from this medium approximately 3 to 4 weeks later and were expanded in RPMI 1640 supplemented with 10% FBS and 300 μg/ml G418. SGP-2-expressing clones were first selected on the basis of the level of SGP-2 message expression. The highest expressing clones were then screened via Western blot for expression of SGP-2 protein.

Immunocytochemical Staining for SGP-2. LNCaP cells were cultured on poly-L-lysine (Sigma Chemical Co., St. Louis, MO)-coated slides and treated with TNF (10 ng/ml). At hourly intervals, slides were washed in PBS and were fixed in 4% neutral buffered formalin at 4°C for 10 min. Immunocytochemical staining for SGP-2 was conducted using a commercial kit (ABC Kit; Vector Laboratories, Burlingame, CA). Normal goat serum was used to block nonspecific binding sites in the slides, which were then incubated in a humidified chamber for 18 h at 4°C with a 1:400 dilution (12.5 μg/ml) of an IgG fraction antisera to SGP-2 (16). Antigen was visualized by subsequent incubations with biotinylated second antibody, avidin-biotin horseradish peroxidase complex, and diaminobenzidine tetrahydrochloride before counterstaining with Gill's hematoxylin.

Negative control slides were processed in an identical manner by substitution of primary antisera with normal rabbit IgG at the same concentration as that used for the primary antisera. No color reaction was observed in all negative control slides. Photomicrographs were taken through an Olympus OM-2 microscope (Olympus Corp., Woodbury, NJ) using Ektachrome 64 T film for color slides (Eastman Kodak, Rochester, NY). Color prints were developed from these slides and kept as the permanent record.

Western Blot Detection of SGP-2 Molecular Forms. Cells were detached from culture flasks by trypsin treatment, washed twice in PBS, and resuspended in SDS sample buffer [50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol, and 0.2% bromophenol blue) and heated to 95°C for 5 min. The protein content of each sample was determined by the method of Lowry *et al.* (28). Electrophoresis was carried out in a 10% polyacrylamide gel at 20 V and 200 mA for 5 h according to the method of Laemmli *et al.* (29). Total protein (100 μg) was loaded onto each lane. Rainbow high molecular weight markers (Amersham, Inc., Arlington Heights, IL) were used to determine molecular weights of SGP-2 forms. Following electrophoresis, proteins were transferred to 0.45 μm nitrocellulose membranes according to the method of Towbin *et al.* (30) using a semidry transfer apparatus (Hoeffer Scientific Instruments, San Francisco, CA) at 10 V and 250 mA for 2.5 h. The transferred membrane was incubated with a blocking solution (5% carnation nonfat dry milk in PBS containing 0.01% sodium azide) for 2 h, followed by incubation with primary antibody against SGP-2 for 18 h at 4°C (1:400 dilution; 12.5 μg IgG/ml in blocking solution; Ref. 16). The membrane was then washed in blocking solution and incubated with a 1:12,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA) for 4 h at room temperature. The immunoreactive bands were visualized by incubation of the membrane with enhanced chemiluminescence detection reagent (ECL Western blotting analysis system; Amersham) according to manufacturer's directions. Following a 1-min incubation, the membrane was placed between plastic sheets and exposed to X-AR2 film (Eastman Kodak, Rochester, NY) for 1 min. Quantitation of the protein band was carried out by means of an automated image analysis system (PDQUEST; Pdi, Huntington Station, NY) to determine the relative integrated density of each band. The relative integrated density of each band was calculated by taking the absorbance multiplied by the surface area of the band and was normalized with the value for the wild-type LNCaP cells, which was assigned a value of 1.0.

Two-Dimensional Gel Electrophoresis and Fluorography. Cells in 6-well plates were labeled for 6 h with 50 μCi/well [^{35}S]methionine (specific activity, 1340 Ci/mmol; Amersham). Following labeling, cells were washed twice with PBS and then scraped from the well with the aid of a rubber policeman. An aliquot of the cell sample was taken for protein determination (28) and for determination of radioactivity by precipitation with an equal volume of 20% trichloroacetic acid, washing three times with 10% trichloroacetic acid and reconstitution in a minimal volume of 4 N NaOH. The remaining sample was solubilized in UREA mix [9 M urea, 4% NP40, 2% LKB Ampholyte (pH 9–11), and 2% DTT] and allowed to stand at room temperature for 30 min before centrifugation (30 min, 100,000 × *g*; Airfuge; Beckman Instruments, Fullerton, CA). The ISO-DALT system of Anderson and Anderson was used in the present study as described previously (3, 31, 32). Isoelectric focusing was conducted in 5% acrylamide tube gels with 9 M urea and a mixture of Ampholytes ranging from pH 3–10. Prefocusing was carried out for 200 V h, followed by isoelectric focusing for 14,000 V h at 800 V. Following focusing, gels were extruded and equilibrated in 138 mM Tris base, 10% glycerol, 2% SDS, and bromophenol blue (pH 6.8). The second dimension was conducted in slab gels consisting of 9–18% linear gradient polyacrylamide gels containing 1% SDS. The electrophoresis buffer contained 24 mM Tris base, 0.2 M glycine, and 1% SDS. Electrophoresis was conducted for 18 h at 180 V. Gels were then fixed in 2.5% sulfosalicylic acid in 20% ethanol and soaked in a water-soluble fluor containing 5% glycerol, dried, and exposed to X-ray film as described previously (3).

Statistics. All numerical data were expressed as mean ± SEM. All determinations were carried out in triplicate. Differences among treatment groups were determined by Student's *t* test. A value of $P < 0.05$ was considered to be statistically significant (33).

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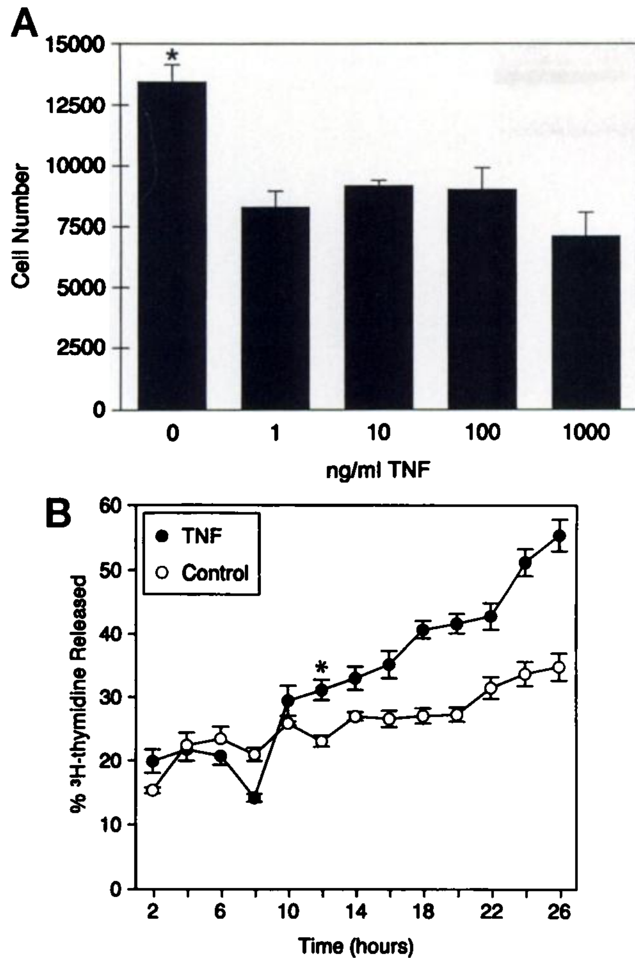

Fig. 1. Effect of TNFα on LNCaP cell death. *A*, LNCaP cells were plated in 24-well plates and were allowed to adhere overnight. Cells were cultured for 48 h in RPMI 1640 supplemented with ITS Plus and varying concentrations of TNF as described in "Materials and Methods." Cells were detached from the culture surface and counted with a Coulter counter to determine cell numbers in each group. Values represent the mean; *bars*, SE; $n = 4$/group. *, $P < 0.05$ compared to cells treated with TNF. *B*, effect of TNF treatment on the release of [^{3}H]thymidine from prelabeled LNCaP cells. *Points* represent the mean; *bars*, SE. $n = 4$/group. *, $P < 0.05$ compared to control without TNF treatment at the same time point. The percentage of thymidine release was significantly greater in TNF-treated cells at all time points after 12 h post TNF treatment.

RESULTS

Effect of TNF on LNCaP Cells. Treatment of LNCaP cells with TNF resulted in a significant inhibition of total cell number. Fig. 1*A* shows the effect of different concentrations of TNF on these cells after 48 h of treatment. The decrease in total cell number was reflected by an increase in cell death, as assessed by the release of radioactivity from cells prelabeled with [^{3}H]thymidine (Fig. 1*B*), detection of DNA fragmentation (Fig. 2), and by trypan blue dye exclusion test (Table 1). The difference in the timing of onset of these events is worth noting. Upon TNF treatment, the onset of DNA fragmentation in LNCaP cells occurred at 6 h, whereas the actual manifestation of cell death, as demonstrated by an increase in [^{3}H]thymidine release and by an increase in trypan blue dye uptake, did not become apparent until 12 h. Therefore, these results indicated that TNF treatment of LNCaP cells would result in cell death after 12 h but that DNA fragmentation was initiated 6 h earlier.

Effect of TNF on Immunocytochemical Staining for SGP-2. Fig. 3 shows the temporal changes in the pattern of SGP-2 staining in LNCaP cells at different intervals following TNF treatment. Prior to TNF treatment, LNCaP cells showed weak, although positive, staining for SGP-2. At 1 h after TNF treatment, the intensity of SGP-2 staining was increased and remained elevated for at least 4 h, following which the intensity reduced gradually. At 6 h of TNF treatment, SGP-2 was not detected in LNCaP cells on the basis of immunocytochemical staining. These results, along with those described above, indicated that there was a transient elevation of SGP-2 staining in LNCaP cells upon TNF treatment, but the staining disappeared at the onset of DNA fragmentation, which was followed by the manifestation of cell death 6 h later.

Effect of Antisense Oligonucleotides to SGP-2 on LNCaP Cells. Four different antisense oligonucleotides were used in the present study. They were designated as antisense 1-21, 22-42, 43-63, and 64-84, according to the sequence of SGP-2 mRNA extending from the start site of the message. Treatment of LNCaP cells with a mixture of these antisense oligonucleotides, at 140 μM, resulted in a significant increase in cell death at 24 h. Lower concentrations of the oligonucleotide mixture showed a concentration-related reduction in cytotoxic effect (Fig. 4). Furthermore, antisense 1-21 was the most effective in LNCaP cytotoxicity. The effectiveness was reduced as the antisense sequences moved away from the start site (Table 2; Fig. 5,

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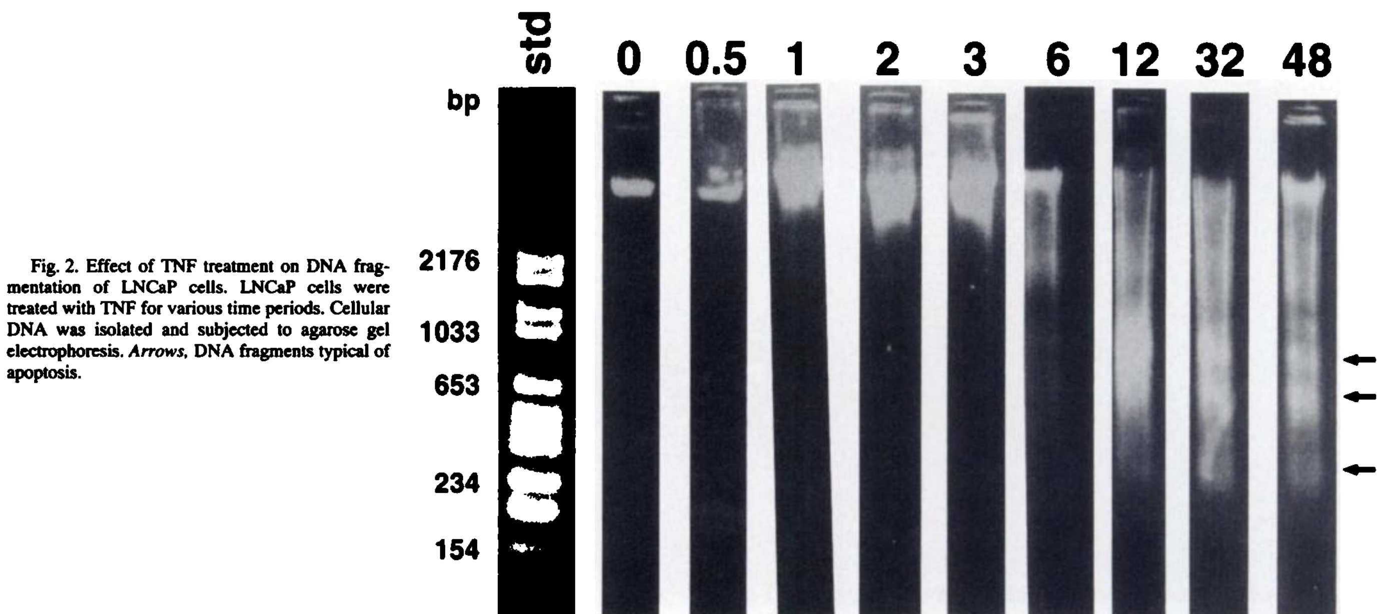

Fig. 2. Effect of TNF treatment on DNA fragmentation of LNCaP cells. LNCaP cells were treated with TNF for various time periods. Cellular DNA was isolated and subjected to agarose gel electrophoresis. *Arrows*, DNA fragments typical of apoptosis.

Table 1 *Effect of TNF on viability of LNCaP cells*

Each value represents mean ± SEM of three separate experiments. Cell viability was assessed by the tryphan blue exclusion test.

	% viable cells	
Treatment (h)	TNF 10 ng/ml	Control (no TNF)
6	95.2 ± 0.54	97.1 ± 0.93
12	93.0 ± 0.45	90.0 ± 1.56
18	40.2 ± 6.27[a]	87.8 ± 1.89
24	35.3 ± 1.94[a]	84.6 ± 2.08

[a] The value is significantly different from the corresponding value in the control (no TNF) group ($P < 0.05$).

A and *C*). Oligonucleotides with a sense sequence (Fig. 5, *B* and *D*) or antisense to an irrelevant gene product such as albumin, at the same molar concentration, had no cytotoxic effect to LNCaP cells (Fig. 5, *A* and *C*).

In order to investigate if treatment with antisense oligonucleotides to SGP-2 had an impact on the ability of LNCaP cells to synthesize SGP-2, cells were treated with respective antisense or sense oligonucleotides for

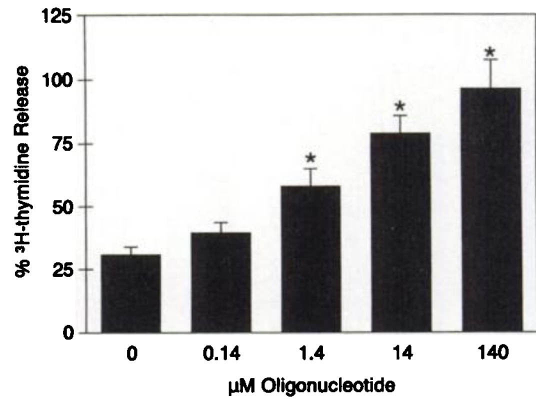

Fig. 4. The percentage of thymidine release from prelabeled LNCaP cells following treatment with oligonucleotides antisense to SGP-2 cDNA. LNCaP cells were incubated with a mixture of antisense oligonucleotides (AS 1-21, AS 22-42, AS 43-63, and AS 64-84) as described in "Materials and Methods" for 24 h. Values represent the mean; *bars*, SE; $n = 4$/group. *, $P < 0.05$ compared to cells treated without oligonucleotide mixture.

Fig. 3. SGP-2 immunocytochemical staining in LNCaP cells at different intervals following TNF treatment. Brown staining indicates cells expressing SGP-2 protein. × 140.

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Table 2 *Effect of transient transfection of LNCaP cells with oligonucleotides with sequence antisense or sense to SGP-2 on cell viability*

Each value represents mean ± SEM of three separate wells. Cell viability was assessed by the trypan blue exclusion test. The percentage of viable cells for the control culture without the addition of oligonucleotides was 97.0 ± 0.14%.

Range of SGP-2 coding sequence	% viable cells	
	Antisense sequence	Sense sequence
1–21	9.0 ± 1.82[a]	93.7 ± 1.01
22–42	39.8 ± 1.91[a]	94.0 ± 1.21
43–63	96.3 ± 0.83	96.5 ± 0.82
64–84	95.2 ± 2.66	96.2 ± 0.90

[a] The value is significantly different from the corresponding value in the sense sequence group at $P < 0.05$.

6 h, together with [^{35}S]methionine. Two-dimensional fluorography of cellular proteins demonstrated that treatment of LNCaP cells with antisense 1-21 resulted in a decline of SGP-2 synthesis as compared to cells treated with the corresponding sense oligonucleotide (Fig. 6).

Characterization of SGP-2 Protein Expressed by Transfected LNCaP Clones. The molecular forms and relative levels of expression of SGP-2 protein produced by the SGP-2-transfected LNCaP cell clones was investigated by Western blot (Fig. 7). Clone SSE, passage 8, produced the highest levels of SGP-2 protein (relative integrated density, 2.41). In addition, this clone produced both precursor and α and β subunits of SGP-2 with molecular weights comparable to native SGP-2 (19). Clone SS7 (Fig. 7, *Lane 2*) produced less SGP-2 protein than control, non-SGP-2 transfected cells, and vector alone transfected cells (Fig. 7, *Lanes 5* and *6*). Clone SS6 produced an intermediate level of SGP-2 protein.

Effect of TNF on SGP-2-transfected LNCaP Cell Clones. Treatment of LNCaP cell clones with TNF resulted in a significant inhibition of total cell number in wild-type, neomycin alone transfected, and in the SGP-2-transfected clones SS6 and SS7. In contrast, the SGP-2-transfected clone SSE was resistant to the cytotoxic effect of TNF (Fig. 8). A trypan blue dye exclusion test confirmed that clone SSE did not undergo significant cell death following TNF administration (Table 3). In contrast to the result in Fig. 8, clone SS6 was found to be resistant to TNF-induced apoptosis when evaluated by trypan blue dye exclusion test. The trypan blue determination was conducted at 18 h after TNF treatment, as compared to the determination of cell

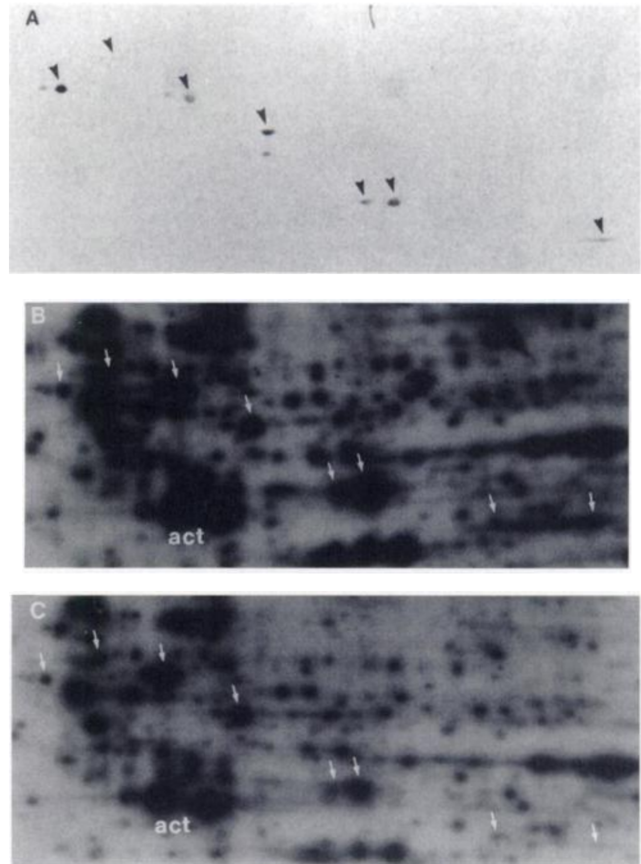

Fig. 6. Effect of antisense oligonucleotide to SGP-2 cDNA on the biosynthesis of SGP-2 protein. *A*, two-dimensional Western blot of LNCaP cells incubated with antisera raised against human SGP-2. *Arrowheads*, reactive peptides. *B* and *C*, LNCaP cells were treated with sense (S 1-21) oligonucleotides (*B*) or antisense (AS 1-21) oligonucleotides (*C*) as described in "Materials and Methods" for 4 h together with 100 μCi/ml [^{35}S]methionine. Cell lysates were prepared, and 70 μg of protein was used for two-dimensional gel fluorography. Exposure time to X-ray film was for 72 h. *Arrows*, peptides reactive with an antibody specific for human SGP-2 by Western blot analysis (see *A*). The synthesis of the *four rightmost* SGP-2 peptides indicated by *arrows* was either significantly reduced or undetectable following treatment with antisense oligonucleotide AS 1-21. *act*, actin; molecular weight, approx. M_r 40,000, is indicated as an internal marker protein for orientation.

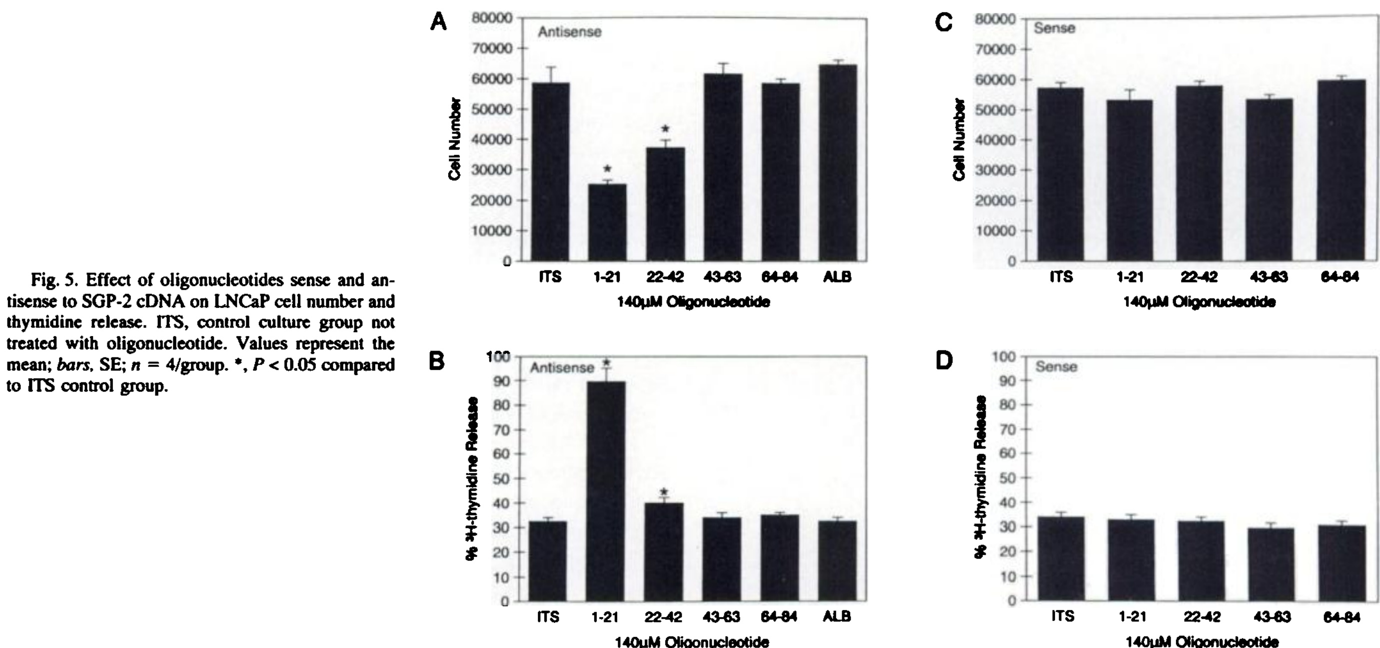

Fig. 5. Effect of oligonucleotides sense and antisense to SGP-2 cDNA on LNCaP cell number and thymidine release. ITS, control culture group not treated with oligonucleotide. Values represent the mean; *bars*, SE; $n = 4$/group. *, $P < 0.05$ compared to ITS control group.

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number, which was determined at 24 h after TNF treatment. The observed differences in resistance to TNF treatment in clone SS6 may, therefore, reflect an ability of this clone to delay the onset of apoptosis.

DISCUSSION

Results of the present study have provided evidence to indicate that an overexpression of SGP-2 in LNCaP cells could protect the cells from the cytotoxic effect of TNF. These findings, coupled with the observation that a depletion in SGP-2 expression is linked to cell death, have allowed us to propose a functional role for SGP-2 in programmed cell death in LNCaP cells. The following considerations warrant further discussion.

TNF is cytotoxic to a large number of cell types including LNCaP cells (20, 34, 35). Its effect is mediated through the interaction with the TNF receptor, a M_r 55,000 protein that contains an approximately 80-amino acid domain near the COOH terminus known as the cell death domain (34). The cellular events related to TNF-induced cell death have been well characterized (35, 36). The effect of TNF on LNCaP cells has also been recognized (20). Results of the present study have demonstrated that LNCaP cells, upon exposure to TNF,

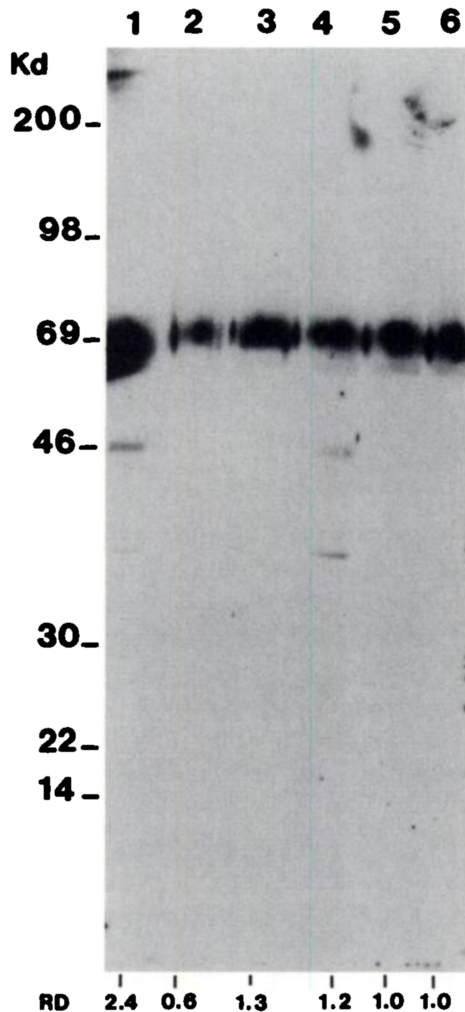

Fig. 7. Western blot analysis of the expression of SGP-2 protein by SGP-2 stably transfected LNCaP clones. *Lane 1*, clone SSE, passage 8; *Lane 2*, clone SS7; *Lane 3*, clone SS6; *Lane 4*, clone SSE, passage 2; *Lane 5*, vector control; *Lane 6*, wild type (parental line). *RD*, relative integrated density of SGP-2 protein expression. The relative density of each band was determined by the product of absorbance multiplied by the surface area of the band and was normalized with the value for the parental line, which was assigned a value of 1.0.

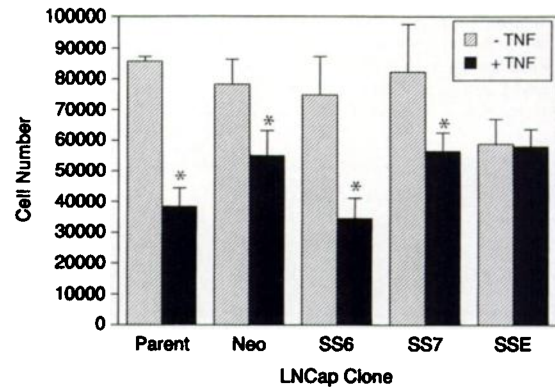

Fig. 8. Effect of TNF treatment on SGP-2-transfected LNCaP clones. LNCaP cells were treated with TNF for 24 h. Cell number was determined by Coulter counter. Values represent the mean; bars, *SE*. $n = 4$/group. *, $P < 0.05$ compared to the same clone not treated with TNF.

Table 3 *Effect of TNF on viability of different clones of LNCaP cells transfected with SGP-2 gene*

Each value represents mean ± SEM of three separate cultures, and cell viability was assessed by the trypan blue exclusion test.

	% viable cells	
LNCaP clones	TNF-treated (10 ng/ml)	Control (no TNF)
Wild type (parental line)	58.3 ± 0.80[a]	87.3 ± 3.65
Vector control (Neo)	61.1 ± 7.36[a]	89.0 ± 1.87
Clone-1 (SS6)	74.8 ± 8.44	84.0 ± 1.16
Clone-2 (SSE)	90.5 ± 2.65	93.8 ± 1.46

[a] The value is significantly different from the corresponding value in the control group at $P < 0.05$.

undergo programmed cell death. Therefore, TNF-induced cell death in LNCaP cells may be considered as an acceptable model for the study of the biology of programmed cell death.

DNA fragmentation has been considered a hallmark of programmed cell death (37, 38). In the present study, this event occurred in LNCaP cells at 6 h of TNF treatment. The actual manifestation of cell death, as demonstrated by an increase in the percentage of trypan blue-positive cells and by an increase in release of previously incorporated [^{3}H]thymidine, did not occur until 12 h after TNF treatment. These events are, therefore, considered as the late manifestations of the effect of TNF. Among the early events are the initial activation and subsequent depletion of SGP-2 expression in LNCaP cells upon exposure to TNF. The event of SGP-2 depletion preceded the onset of DNA fragmentation in these cells and may be responsible for the initiation of the cell death process. This possibility was proposed because, in the present study, cell death could be induced by treatment of LNCaP cells with oligonucleotide sequences antisense to SGP-2. The above treatment also resulted in a diminution of SGP-2 biosynthesis, which was independent of the effect of TNF. Therefore, in these two different treatments, TNF-induced cytotoxicity and antisense-mediated cytotoxicity; a depletion of SGP-2, rather than its expression, has been the common event associated with the onset of cell death in LNCaP cells.

As indicated above, the present system of TNF-induced cytotoxicity in LNCaP cells is characterized by an initial transient elevation of SGP-2 expression. Although the biological significance of this transient event is unclear, it is apparent that this event represents a distinct response of LNCaP cells to the stress imposed by TNF treatment. A similar pattern of transient elevation of SGP-2 expression has been noted in the rat prostate and in the rat kidney upon receiving cell death stress signals (3, 4, 13). In the case of the rat prostate during castration-induced cell death, unlike the TNF-induced cytotoxicity in

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LNCaP cells, the subsequent SGP-2 depletion has not been unequivocally observed. Rather, a significant decline in tissue levels of SGP-2 was realized (3, 11). The difficulty in demonstrating a complete depletion of SGP-2 expression in the rat prostate model could be attributed to the complex *in vivo* system in which cell-to-cell interaction is taking place in a heterogenous fashion (7, 11). Thus, events such as the onset of cell death, do not occur synchronously to allow observation of a complete depletion of SGP-2. It is now apparent that the transient elevation in SGP-2 expression may represent a cellular response to cell death signals but is not directly linked to cell death. SGP-2 depletion, on the other hand, appears to be directly linked to cell death, at least in LNCaP cells.

Finally, the phenomenon of an overexpression of SGP-2 resulting in resistance to TNF-induced cell death in LNCaP cells has not been reported before. The level of SGP-2 protein expressed by the clones studied suggested a correlation between the level of SGP-2 protein expression and degree of resistance to TNF-induced apoptosis. Clone SSE, which produced the highest levels of SGP-2 protein, was resistant to TNF-induced apoptosis as measured by two independent parameters. In contrast, clone SS7, which expressed SGP-2 protein at a level below that of the non-SGP-2-transfected and vector alone transfected control cells, was not resistant to TNF-induced apoptosis. These observations have led us to propose the hypothesis that SGP-2 overexpression can prevent cell death, whereas SGP-2 depletion is responsible for the onset of cell death in LNCaP cells. At present, the mechanism of the effect of SGP-2 overexpression and depletion on LNCaP cells remains unclear. Whether these actions are related to those of the established gene products known to modify cell death, such as the bcl-2 and ced families (39, 40), will be the subject of our future investigations. It is anticipated that as further information becomes available, the above hypothesis may be revised.

In summary, results of the present study have established evidence to demonstrate that an overexpression of SGP-2 in LNCaP cells leads to resistance to TNF-induced cytotoxicity. These results also indicate that TNF-induced cell death in LNCaP cells is characterized by an initial transient activation of SGP-2 expression. Current evidence suggests that depletion of SGP-2 in LNCaP cells is responsible for the onset of cell death. These results have provided an opportunity to assign a functional role for SGP-2 in TNF-induced cell death in LNCaP cells.

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