TIMP-3 INDUCES CELL DEATH BY STABILIZING TNF-α RECEPTORS ON THE SURFACE OF HUMAN COLON CARCINOMA CELLS

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Matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) regulate the structural integrity of the extracellular matrix (ECM). Constitutive expression of human TIMP-3 in human DLD colon carcinoma cells renewed serum-responses and inhibited tumour formation in nude mice. To elucidate the mechanism of TIMP-3-mediated tumour suppression, we compared parental DLD and TIMP-3 expressing DLD cells (TIMP-3/DLD), finding them to be significantly different. TIMP-3/DLD cultures have fewer mitotic cells, are delayed in G_1, and die after serum starvation. TIMP-3/DLD conditioned media activates cell death on fibroblast cells. The cell death induced by serum starvation and conditioned media was inhibited by 70%, in the presence of neutralizing tumour necrosis factor α (TNF-α) antibody. TIMP-3/DLD whole cell lysate contained p55 TNF-α receptor, while vector/DLD lysate had p55 TNF-α receptor and p46 soluble TNF-α inhibitor. Vector/DLD conditioned media had p46, while no soluble TNF-α receptor was detected in TIMP-3/DLD conditioned media. In addition, FACS analysis revealed that TIMP-3/DLD cells have more TNF-α surface binding sites, suggesting a direct correlation between TIMP-3 expression and surface receptors. The mechanism of tumorigenic reversion induced by TIMP-3 in DLD cells may involve protection of receptors from the proteolytic activity of MMPs. Putative TIMP-3-mediated inhibition of MMPs restores the TNF-α p55 signalling pathway and the carcinoma cell is killed by autocrine TNF-α. Thus, DLD cells have specific ECM MMPs that cleave cytokines and cytokine receptors. TIMP-3 specifically inhibits MMPs involved in receptor shedding.

The extracellular matrix (ECM) is an important cellular compartment that is especially active during development, and deregulated activity is thought to play a role in the aetiology of diseases such as cancer and arthritis.[1–3] Matrix metalloproteinases (MMPs) are a family of secreted zinc-containing proteolytic enzymes that localize to and impact ECM remodelling.[4] The activity of MMPs is regulated at three distinct levels, transcription of the gene, proenzyme secretion and activation, and through specific protein interactions with tissue inhibitors of metalloproteinases (TIMPs). TIMPs bind to enzymatically active MMPs with a 1:1 molar stoichiometry inhibiting proteolysis.

The TIMP gene family has four members which have 30–40% amino acid sequence homology, 12 conserved cysteines suspected to form critical intramolecular disulfide linkages,[5] and are expressed from distinct genes.[6] TIMP-3 is unique in having a high binding affinity for the ECM[7,8] and it inhibits several known MMPs.[9] Human and murine TIMP-3 have been cloned and sequenced by several groups.[7–12] TIMP-3 expression is downregulated in neoplastic murine JB6 cells and other tumour cell lines,[8] inhibits MMP activity,[7] and is upregulated during G_1 phase of the cell cycle by signals that induce mitogenesis, differentiation, and senescence.[10] The clinical significance of TIMP-3 was realized from studies with the eye. TIMP-3 mutations correlate to Sorby's fundus dystrophy[13] and tissue isolated from a retinal disease, simplex retinitis pigmentosa, have elevated levels of TIMP-3 mRNA.[14]

TIMP-3 was cloned by mRNA differential display from a murine cell line model (JB6) of multi-step carcinogenesis.[8] In JB6 cells, TIMP-3 was expressed in multiple independent preneoplastic but not neoplastic cell lines,[8,15] suggesting a link between neoplastic

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TABLE 1. TIMP-3 expression in DLD carcinoma cells decreases the percentage of mitotic cells

Cell line	TIMP-3 expression	% Cells in cell cycle phase					
		Inter	Pro	Met	Ana	Telo	% Mitosis
Vector/DLD	ND	76	12	7	2	3	24
TIMP-3/DLD	Low	81	10	5	2	2	19
TIMP-3/DLD	High	91	5	2	1	1	9
TIMP-3/DLD SS	High	96	2	1	0.4	0.4	4

TIMP-3 expression was determined by Western blotting.[16] Low TIMP-3/DLD cells express only 5% of the TIMP-3 protein found in the TIMP-3/DLD cells.[16]
Cells (10^5) were seeded on glass coverslips and grown in MEM supplemented with 15% FCS or serum-starving (SS) 0.5% FCS media for 48 h to 50–70% confluence. Cultures were washed with PBS, fixed with cold methanol/acetone, stained with DAPI, and approximately 600 cells per coverslip were scored for cell cycle distribution using UV microscopy. The number was averaged from three independent experiments performed in duplicate (approximately 3600 total cells were scored per cell line). ND, not detected by immunoblot analysis.

transformation and the downregulation of TIMP-3. To evaluate the role of TIMP-3 in human cancers, we transfected a human TIMP-3 expression plasmid into human DLD colon carcinoma cells, which have no detectable endogenous TIMP-3 expression. TIMP-3/DLD transfectants regain serum-responsiveness, no longer form tumours in nude mice, and form large necrotic aggregates in suspension culture.[16] In this report, we demonstrate that a TIMP-3/DLD transfectant expressing high levels of TIMP-3, and a transfectant expressing levels of TIMP-3 that are barely detectable by Western blotting (low TIMP-3/DLD) are sensitive to programmed cell death. Cell death is activated by TIMP-3 via protection of TNF-α receptors. Protection may result from the TIMP-3-mediated inhibition of MMP activity.

RESULTS

TIMP-3 expression delays cell cycle progression of DLD colon carcinoma cells in G_1 phase

We have previously shown that DLD carcinoma transfectants overexpressing TIMP-3 were growth arrested under low serum growth conditions.[16] To characterize TIMP-3-mediated effects on proliferation and cell cycle distribution, we determined the percentage of TIMP-3/DLD and control cell lines that are in specific mitotic phases during log phase growth. The TIMP-3/DLD transfectant had a reduced percentage of cells in the population with mitotic chromosomes. As summarized in Table 1, vector/DLD cultures had 76% of the population in interphase with 24% distributed in mitotic phases. TIMP-3/DLD had 91% of the cells in interphase with 9% in mitosis. The low TIMP-3/DLD cells had 81% of the population in interphase and 19% in mitosis. If TIMP-3/DLD cells were serum starved [0.5% fetal calf serum (FCS)] for 48 h, the majority of the cells (96%) were in interphase with only 4% in mitosis (Table 1). These data suggest that TIMP-3 expression delays progression of the cell cycle in a dose-dependent manner that is enhanced at more stringent conditions of low serum.

To determine the average cell cycle distribution of TIMP-3 expressing and control cell lines, FACS cell cycle analysis was performed. Density-inhibited cultures were incubated in 15% or 0.5% FCS MEM for 48 h. In addition, actively growing 60% confluent cultures were analysed. Both vector/DLD and TIMP-3/DLD cells gave similar FACS profiles when contact inhibited and incubated in 15% FCS (Table 2). TIMP-3/DLD cells stressed by serum-starvation (0.5% FCS) for 48 h were shifted out of G_2/M and S into G_1. The vector/DLD distribution after 48 h of serum starvation (0.5% FCS) was similar to both vector/DLD and TIMP-3/DLD grown in 15% FCS, while serum-starved TIMP-3/DLD cells are delayed in G_1 (Table 2). Log phase growing TIMP-3/DLD cells had a

TABLE 2. FACS cell cycle analysis: TIMP-3 expression and serum-starvation delay G_1

Cell line	Growth status	% Population in phase		
		G_1	S	G_2/M
	Confluent			
Vector/DLD	15% FCS	58	25	17
	0.5% FCS	63	21	16
TIMP-3/DLD	15% FCS	55	24	21
	0.5% FCS	76	15	9
	Log phase			
Vector/DLD	15% FCS	52	27	21
TIMP-3/DLD	15% FCS	66	20	14

Cells (10^6) were plated in 150-mm dishes and grown in 15% FCS MEM to dentisy-inhibited monolayers or until 60% confluent. Fresh media with 15% or 0.5% FCS was applied to density-inhibited cultures and they were incubated at 37°C for 48 h. Cells were removed from the dish with trypsin, washed in isotonic PBS, suspended in 0.3 ml PBS, and added to 2 ml of Thornthwaite lysis buffer. RNase A (200 μg) was added for 15 min at room temperature and PI (50 μg/ml) was added to the suspension. The preparations were incubated in the dark for 1 h at room temperature and stored at 4°C for 20–50 h before flow cytometric analysis. The data presented in the table was one experiment out of three, all gave similar results.

significant percentage of the population delayed in G_1 compared to control cells, with fewer cells in G_2/M and S (Table 2). The reduction of [^{3}H]thymidine incorporation observed with TIMP-3/DLD cells grown in 0.5% FCS[16] is due to a TIMP-3-mediated G_1 delay. These results suggest that under conditions of serum starvation and rapid proliferation, TIMP-3 expression delays G_1 phase of the cell cycle in malignant DLD colon carcinoma cells.

TIMP-3 expression sensitizes DLD cells to cytotoxic signals

During the course of our experiments it was observed that the morphology of serum-starved TIMP-3/DLD cells was similar to TNF-α treatment of murine fibroblasts (increased light refraction due to cell rounding, shrinkage, and lysis). To examine the nature of serum-starved TIMP-3/DLD cell death, cytotoxicity assays and DNA fragmentation analysis were performed.

Exposure of TIMP-3/DLD cells to H_2O_2 and serum-starvation resulted in cytotoxicity (Fig. 1) that was measured by nuclear uptake of propidium iodide (PI). This assay has been used to detect cytoplasmic membrane degeneration, an early TNF-α-mediated response.[17] Figure 1 shows the cellular morphology of killing after a 50-h treatment of TIMP-3/DLD confluent monolayers with H_2O_2 (A and B), serum-starvation in 0.5% FCS media (C and D), but not with 15% FCS media (E and F). Hydrogen peroxide treatment and low serum growth conditions induce cell rounding, shrinkage, and lysis (B and D, compared to F) and PI uptake into nuclei (A and C, compared to E). Serum-starvation of low TIMP-3/DLD and vector/DLD cell lines did not cause significant cell rounding, shrinkage, or lysis, and did not lead to PI uptake (data not shown). TIMP-3 expression in DLD cells induced programmed cell death after exposure to stressful conditions, as illustrated by peroxide treatment or serum withdrawal. Data from several experiments are summarized in Table 3. The vector/DLD cells were resistant to stress-induced killing, while TIMP-3/DLD cells were sensitive. The low TIMP-3 expressing DLD cell line gave an intermediate level of cytotoxicity. Hydrogen peroxide treatment induced substantial TIMP-3/DLD cell death even under conditions of high serum (Table 3).

To confirm and extend our PI uptake and morphological observations, that constitutive TIMP-3 expression induced cell death in DLD cells, we performed a DNA fragmentation analysis. Confluent monolayers were incubated for approximately 60 h with either 15% or 0.5% FCS media. DNA was purified and characterized by agarose gel electrophoresis (Fig. 2). Serum-starved TIMP-3/DLD cells showed a major band of DNA (RNase A treatment had no effect on this band) migrating quickly through the gel that may represent the 180 base pair fragment often seen with apoptosis.[18] This band was not found after serum-starvation of vector/DLD and low TIMP-3/DLD, nor was this DNA degradation found in the three cell lines incubated with 15% FCS media (Fig. 2A). A time course of TIMP-3/DLD serum-starvation (0.5% FCS) demonstrated enhanced DNA fragmentation with time and after 60 h, indications of laddering were visible on gels, with increments of approximately 180 base pairs (Fig. 2B). This pattern of DNA laddering and smearing (associated with necrosis accompanying apoptosis) is similar to that observed after TNF-α treatment of fibroblasts[19,20] and epidermal cells.[21]

Conditioned media from TIMP-3/DLD cultures activates cell death on TNF-α-sensitive fibroblasts, via the TNF-α receptor signalling pathway

Since the morphology and nuclear PI uptake of serum-starved TIMP-3/DLD cells (Fig. 1) and the fragmentation of DNA (Fig. 2) were reminiscent of TNF-α cytotoxicity, we tested TIMP-3/DLD conditioned media (48 h) for killing activity on TNF-α-sensitive fibroblasts. TIMP-3/DLD conditioned media induced a time-dependent cytotoxicity when applied 1:1 (V/V) with 15% FCS media to confluent monolayers of NIH 3T3 cells. Again the cytotoxicity was similar to that observed after treatment with TNF-α (Table 4). Conditioned media from vector/DLD and low TIMP-3/DLD cells did not have killing activity (data not shown).

The biological consequences of serum-starving TIMP-3/DLD cells, as discussed above, were reminiscent of TNF-α-induced cytotoxicity. In addition, TNF-α exhibited differential killing activity on the TIMP-3/DLD cells compared to the vector/DLD cells, showing an 8-fold greater level of cytotoxicity at 200 ng/ml (Fig. 3). The possibilities for the killing mechanism include a direct role for TNF-α. To distinguish between a mechanism dependent on or independent of secreted TNF-α, neutralizing antibodies specific for human and murine TNF-α were added at the beginning of the serum starvation. The 75% cytotoxicity observed after 48 h of serum-starvation of the TIMP-3/DLD cells was reduced to 18% (a 76% decrease) when neutralizing anti-human TNF-α antibody was included (Table 4). TIMP-3/DLD conditioned media-induced killing was also inhibited by neutralizing anti-human TNF-α antibody (Table 5). Addition of neutralizing anti-murine TNF-α antibody or anti-human interleukin 1β (IL-1β) antibody to the incubation had no effect on cytotoxicity. These experiments suggest that TNF-α secretion plays an important role during the TIMP-3/DLD cytotoxicity

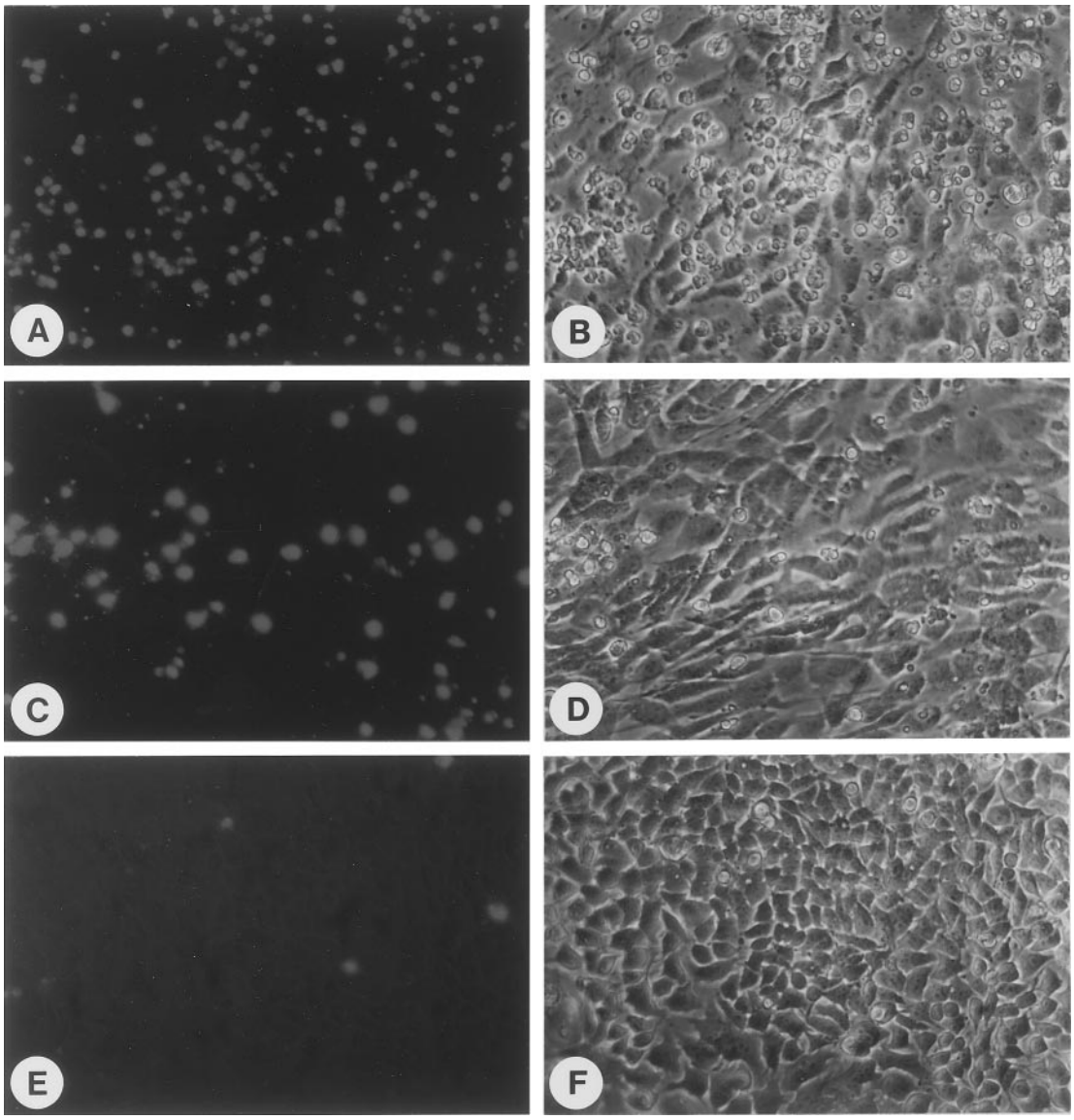

Figure 1. Hydrogen peroxide and serum starvation activate TIMP-3/DLD programmed cell death.

Fluorescent (A, C, and E) and phase contrast photomicrographs (B, D, and F) of TIMP-3/DLD cells illustrate how hydrogen peroxide (A and B) and serum starvation (C and D) induce nuclear PI uptake (A and C) and apoptotic-like morphology (B and D) compared to cells grown in 15% FCS media (E and F). Panels A and B, C and D, and E and F are identical fields of cells. TIMP-3/DLD cells were seeded on glass coverslips at 1×10^5 cells/ml in 15% FCS MEM and grown to confluence. The media was replaced with 15% FCS MEM plus 20 μM hydrogen peroxide (A and B), 0.5% FCS MEM (C and D), and 15% FCS MEM (E and F). The cultures were incubated at 37°C for 50 h, living cells were stained with 1 μM PI, and photographed using fluorescent and phase contrast filters at 100×.

induced by serum-starvation and that induced by TIMP-3/DLD conditioned medium.

Our hypothesis was that TIMP-3 might upregulate TNF-α expression, synthesis, or release from DLD cells. Other reports indicate that MMPs are involved in regulating TNF-α processing and release from various cell lines.[22,23] In the DLD carcinoma model, TIMP-3 seems to increase TNF-α levels in conditioned media, since only TIMP-3 expressing TIMP-3/DLD cell conditioned media induced neutralizing antibody inhibitable cell death on indicator cells (Tables 4 and 5). To examine TNF-α gene expression, Northern blot analysis of total RNA isolated from vector/DLD, low TIMP-3/DLD, and TIMP-3/DLD cell lines, grown under conditions of 15% and 0.5% FCS, was performed. The analysis showed that TNF-α mRNA levels were similar in all three cell lines under all growth conditions tested when standardized against actin mRNA levels on the same blot (data not shown). The amount of TNF-α protein present in cell lysate and conditioned medium was estimated by Western blot, enzyme-linked immunosorbent assay (ELISA),

TABLE 3. Induction of programmed cell death in TIMP-3 expressing DLD colon carcinoma cells

Cell line	Percent FCS media	PI staining % Dead cells
Vector/DLD	15.0	1.6 (0.6)
Vector/DLD	0.5	4.0 (1.5)
Low TIMP-3/DLD	15.0	2.5 (0.4)
Low TIMP-3/DLD	0.5	18.0 (2.6)
TIMP-3/DLD	15.0	3.2 (0.7)
TIMP-3/DLD	0.5	60.3 (13.2)
TIMP-3/DLD + H_2O_2	15.0	67.4 (9.2)
TIMP-3/DLD + H_2O_2	0.5	81.0 (16.7)

Cells were seeded on glass coverslips at 2×10^5 cells/ml and grown to confluence. The media was replaced with either 15% or 0.5% FCS MEM and the cultures were incubated at 37°C for 60 h. In some cultures hydrogen peroxide was added to 20 μM. Approximately 3000 total cells per cell line and treatment were scored for nuclear PI uptake by fluorescent microscopy. The data was the average of four separate determinations performed in duplicate. Standard deviation is in parenthesis.

and immunoprecipitation. All three methods of protein quantification suggested that similar levels of TNF-α were present in conditioned media (30–60 pg/ml) and whole cell lysate (60–100 pg/ml) from all three cell lines grown in 0.5% or 15% FCS media (data not shown). The results suggest that TNF-α secretion differences cannot explain the differential cytotoxicity observed with the transfectant cell lines.

TNF-α receptor analysis

TNF-α availability is not the primary factor controlling TIMP-3/DLD serum-starvation cytotoxicity and since TIMP-3 localizes to the ECM,[1,8,16] we assayed each cell line for TNF-α receptors by Western blot analysis and flow cytometry. Cell lysate and concentrated (10×) conditioned media were prepared from TIMP-3/DLD and vector/DLD cells. Immunoblots were stained with anti-human TNF-α receptor I antibody. Figure 4 shows the result from an immunoblot. The conditioned media (M) from vector/DLD cells had one TNF-α receptor band migrating at approximately 46 kDa (Fig. 3, lane 3) with a smear of degraded protein below. Cell lysate (L) from vector/DLD had two TNF-α receptor specific bands, one migrating at 55 kDa and another at 46 kDa (Fig. 3, lane 4). The 55 kDa band is the TNF-α receptor that signals lethal damage into cells, while the 46 kDa protein is a soluble proteolytic cleavage product of the p55 TNF-α receptor.[24] p46 was originally purified from human urine as a soluble TNF-α inhibitor.[25] TIMP-3/DLD cell lysate (L) had one band migrating at 55 kDa, with no proteolytic cleavage products (Fig. 3, lane 2). The TIMP-3/DLD concentrated conditioned media had no discernable bands (Fig. 3, lane 1). Equal loading of protein samples was monitored by staining the immunoblots with ponceau S solution before blocking. The immunoblot for the low TIMP-3/DLD cell line was similar to the vector/DLD cell line

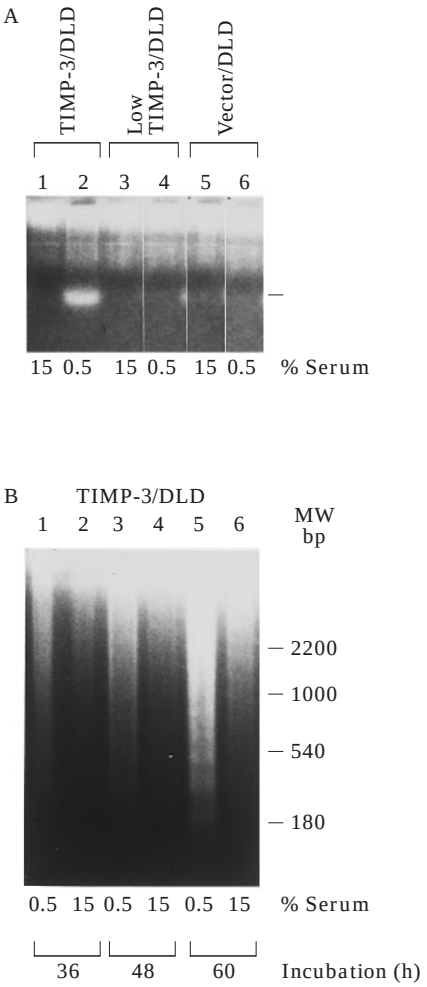

Figure 2. DNA degradation detected after serum-starvation of TIMP-3/DLD cells.

A: Confluent monolayers of TIMP-3/DLD, low TIMP-3/DLD, and vector/DLD cells were incubated in 15% or 0.5% FCS MEM for 57–60 h and DNA was purified. Approximately, 8 μg of DNA isolated from TIMP-3/DLD (lanes 1 and 2), low TIMP-3/DLD (lanes 3 and 4), and vector/DLD (lanes 5 and 6) cells was loaded on 1.4% agarose minigels, run at 70 volts for 10 min, and photographed. Lanes 1, 3, and 5 were from cultures incubated in 15% FCS MEM, while lanes 2, 4, and 6 were from 0.5% FCS MEM cultures. B: Serum-starvation time course of TIMP-3/DLD cells scoring for DNA fragmentation. Approximately 10 μg of DNA was loaded on 1.4% agarose minigels. Lanes 1 and 2 were incubated for 36 h, lanes 3 and 4 for 48 h, and lanes 5 and 6 for 60 h. Lanes 1, 3, and 5 were from cultures incubated in 0.5% FCS MEM, while lanes 2, 4, and 6 were incubated in 15% FCS MEM. Cells were grown in 150-mm dishes to confluence and the media was changed to either 0.5% or 15% FCS media for the indicated times. The cultures were washed with PBS and the cells removed from the dishes with trypsin. Both adherent and floating cells were combined after PBS washing. The gels were run at 60 V for 1.5 h and photographed.

(data not shown). These data indicate that TIMP-3 expression inhibits proteolytic cleavage and shedding of p55 TNF-α receptors. If cleavage and shedding of the p55 TNF-α receptor are impeded by TIMP-3 expression, TIMP-3/DLD cells should have more TNF-α surface binding sites. The relative number of

TABLE 4. TIMP-3/DLD cell-conditioned media is cytotoxic to NIH 3T3 fibroblasts

Time (h)	PI staining percent dead cells 24	48
Conditioned media	37 (4.1)	75 (6.2)
Media	4 (1.1)	6 (0.8)
Media + TNF-α	17 (2.3)	87 (7.7)
Conditioned media + αTNF-α	7 (1.9)	18 (3.4)

Media containing 15% FCS was applied to TIMP-3/DLD cultures and incubated for 50–60 h. The conditioned media was clarified by centrifugation at 25 000 × ***g*** for 30 min. NIH 3T3 cells (10^5) were seeded on glass coverslips in 35-mm dishes and grown to near confluence. The TIMP-3/DLD conditioned media was mixed with an equal volume of fresh 15% FCS media and applied to the cultures. The TNF-α concentration was 100 ng/ml. Excess neutralizing anti-human TNF-α antibody (αTNF-α, 2 mg/ml) was included in some experiments. Percentage of dead cells was determined by PI exclusion, by randomly counting 400–500 cells on each coverslip at 24 and 48 h. The data was the mean and standard deviation (in parenthesis) from at least four experiments performed in duplicate. Conditioned media from vector/DLD and low TIMP-3/DLD cultures was not cytotoxic.

TNF-α binding sites on the surface of TIMP-3/DLD and control cell lines was determined by flow cytometry.

As shown in Figure 5, the TIMP-3/DLD cells have significantly more surface TNF-α binding sites compared to control cells. Mobility shift to the right, in the presence of anti-human TNF-α receptor I antibody, is a direct measurement of the average number of TNF-α receptors found on the cell surface. Relative mean intensity (RMI) is the total absorbance

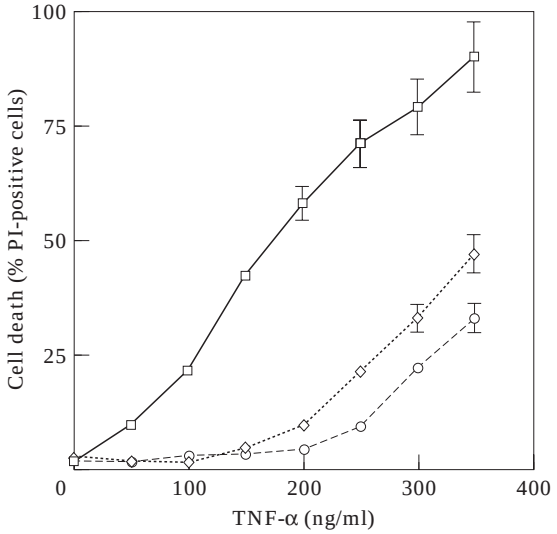

Figure 3. TIMP-3/DLD cells are sensitive to TNF-α-mediated cell death.

Cells were seeded on glass coverslips (1×10^5) in 35-mm dishes and incubated for 10 h. Human TNF-α was added to duplicate dishes at various concentrations, the cultures were incubated at 37°C for 30 h, PI (1 μM) was added, and the cultures were scored for nuclear PI uptake. The experiment was repeated two times and error bars indicate standard deviation. (□), TIMP-3/DLD; (◇), low TIMP-3/DLD; (○), vector/DLD.

TABLE 5. Neutralizing anti-human TNF-α antibody inhibits serum-starved TIMP-3/DLD programmed cell death

Treatment	PI Staining percent cell death DLD cell lines Vector	Low TIMP-3	TIMP-3
15% FCS	2	3	2
0.5% FCS	8	17	70
0.5% FCS + αTNF-α human	10	8	19
0.5% FCS + αTNF-α mouse	9	18	65
0.5% FCS + αIL-1β human	8	20	73

Cells (4×10^5) were seeded on glass coverslips in 35-mm dishes and grown to confluence. The media was changed to either 15% FCS, 0.5% FCS, or 0.5% FCS plus 2 mg/ml neutralizing antibody. The cultures were incubated for 48 h and scored for cell death by PI exclusion. The data presented is a representative experiment which was repeated three additional times with similar results.

recorded by the flow cytometer. Cell sorting of the vector/DLD cell line showed a slight mobility shift to the right (Fig. 5, A), with a RMI of 7.1. Low TIMP-3/DLD cells were shifted further to the right (Fig. 5, B) and a RMI of 21.5 was recorded. The TIMP-3/DLD cell line was shifted prominently to the right and a RMI of 44.6 was recorded by flow cytometry (Fig. 5, C). The analysis demonstrates that TIMP-3/DLD cells have approximately six-fold and two-fold, respectively, more cell surface receptor binding sites for TNF-α compared to vector/DLD and low TIMP-3/DLD cell lines. The p55 TNF-α receptor may, therefore, be the controlling determinant mediating TIMP-3 induced programmed cell death in DLD carcinoma cells.

DISCUSSION

The findings of this report include: (1) TIMP-3 induction of a G_1 delay and programmed cell death; (2) DLD cell secretion of TNF-α is not altered by TIMP-3 expression; (3) TIMP-3 protects TNF-α receptors from proteolytic cleavage, reconstituting the

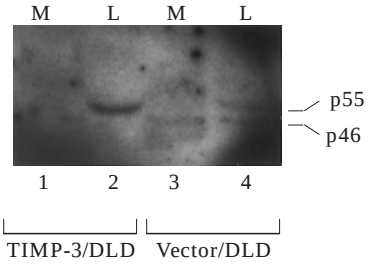

Figure 4. Western blot analysis of TNF-α receptors.

Concentrated conditioned media (M) and cell lysate (L) were prepared from TIMP-3/DLD (lanes 1 and 2) and vector/DLD (lanes 3 and 4) cultures grown to confluence in 15% FCS MEM. The relative mobility of the p55 TNF-α receptor and the p46 TNF-α soluble inhibitor are indicated on the right.

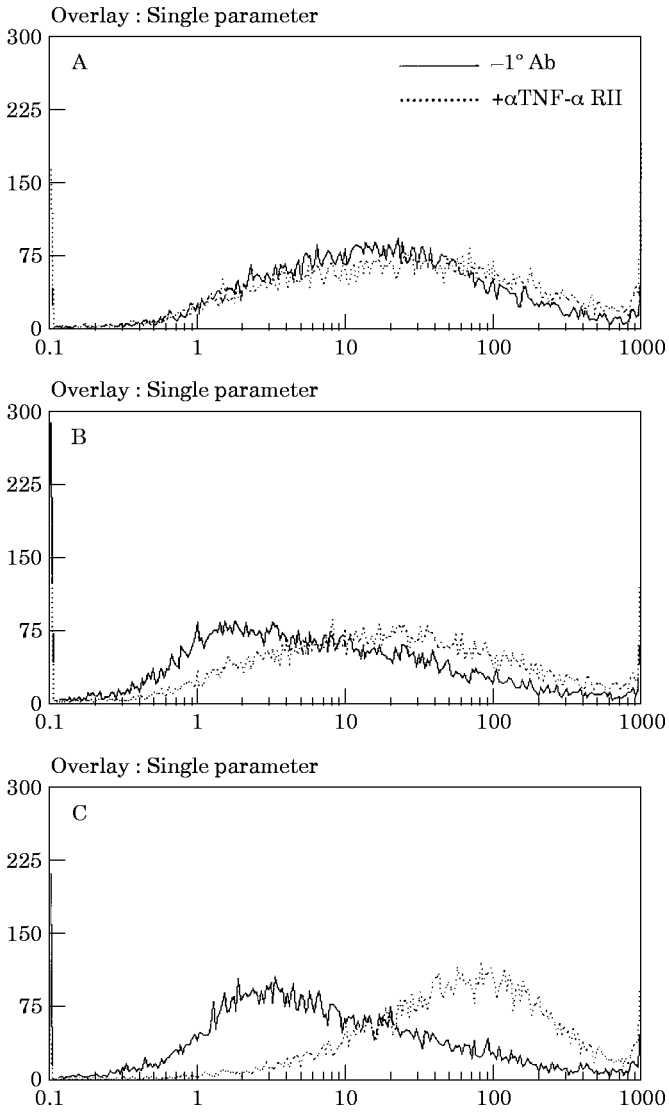

Figure 5. TIMP-3/DLD cells have more p55 TNF-α surface receptors.

Actively growing cultures of vector/DLD (A), low TIMP-3/DLD (B), and TIMP-3/DLD (C) cell lines were harvested and prepared for FACS analysis as described in the Methods Section. The dotted line represents cells treated with primary goat anti-human soluble TNF-α receptor I antibody, secondary biotin-labelled rabbit anti-goat IgG antibody, and PE-labelled streptavidin. The solid line was cells treated in the same fashion omitting the anti-human TNF-α receptor I primary antibody. Contact inhibited cultures gave similar results.

p55 TNF-α signal transduction pathway in DLD carcinoma cells.

TIMP-3 expression delays the DLD carcinoma cell cycle in G_1 (Tables 1 and 2). Perhaps TIMP-3 is involved in the regulation of a cell cycle control point. The transformed DLD cells revert to a non-transformed phenotype after constitutive TIMP-3 expression. FACS cell cycle analysis comparing vector/DLD with TIMP-3/DLD cells showed substantial differences in growth properties and cell cycle distribution during serum-starvation. Vector/DLD shifted only 5% of the population out of S and G2/M (from 58% to 63%), while TIMP-3/DLD shifted 21% (from 55% to 76%) of the population into G_1 (Table 2), reinforcing that DLD cells proliferate by a growth factor-independent mechanism. TIMP-3 expression causes DLD cells to revert to a growth factor-dependent phenotype. TIMP-3 expression in WI-38 and HL-60 cells is maximum and restricted to mid-late G_1, after entering S phase TIMP-3 levels dramatically decrease.[10] Our experiments show that TIMP-3/DLD cells have an altered cell cycle distribution, spending more time in G_1, and undergo significant G_1 delays in response to serum starvation. These results clearly demonstrate that TIMP-3 expression alters the cell cycle program of DLD carcinoma cells.

TIMP-3-mediated cell death induced by serum starvation is reminiscent of both apoptosis and necrosis. TNF-α can activate both forms of cell death[20] depending on the cell line or tissue, and often apoptosis and necrosis occur simultaneously. Nuclear uptake of PI is one method of measuring TNF-α-mediated apoptosis.[17,21] DNA fragmentation analysis resulted in ladders and smearing, characteristic of apoptosis and necrosis, respectively. By several criteria including morphology, PI uptake (Fig. 1), and DNA laddering (Fig. 2), the cell death observed after serum-starvation of TIMP-3/DLD cells is a combination of apoptosis and necrosis.

How can overexpression of TIMP-3, an ECM binding MMP inhibitory protein, induce DLD colon carcinoma cell death and transmit killing activity in conditioned media? The present studies suggest that TIMP-3 may function as an MMP inhibitor,[16] protecting TNF-α receptors from proteolytic cleavage and/or shedding mediated by unidentified MMPs. Since intact p55 receptors are necessary to transduce the TNF-α killing signal,[26] TIMP-3-mediated protection of p55 TNF-α receptors cause DLD cells to become sensitive to TNF-α-mediated programmed cell death.

TNF-α is synthesized as a 26-kDa precursor anchored in the cytoplasmic membrane and is proteolytically cleaved at the cell surface by a Zn^{2+}-dependent endopeptidase (TNF-α converting enzyme, TACE) related to MMPs,[22,23] generating the mature secreted 17-kDa form of TNF-α.[27] Similarly, both TNF-α receptors (p55 and p80) are processed at the cell surface by proteolytic cleavage (shedding), releasing a soluble TNF-α-binding receptor fragment, p46[28,29] that antagonizes the biologic activity of TNF-α.[30] Activated T cells process TNF-α and TNF-α receptor concurrently,[31] since a synthetic hydroxamic acid-based inhibitor of the TNF-α processing protease, TNF-α protease inhibitor (TAPI)[32] blocks both TNF-α and TNF-α p80 receptor cleavage.[33] These reports

demonstrate that a chemical inhibitor of MMP activity blocks both shedding of the 80-kDa TNF-α receptor and processing of pro-TNF-α to mature TNF-α. In a recent study by Mullberg *et al*.,[34] TAPI was shown to inhibit shedding of the soluble forms of IL-6 and p55 TNF-α receptors. In our experiments, TIMP-3, a natural MMP inhibitor, blocked shedding and proteolytic processing of the 55-kDa TNF-α receptor, but had no effect on TNF-α processing. This indicates that TIMP-3 has greater specificity for metalloproteinases than does TAPI. In the DLD cell line system there must be at least two MMPs, one that cleaves TNF-α receptors (this MMP is modulated by TIMP-3) and one that processes TNF-α (this MMP is not modulated by TIMP-3). TIMP-3/DLD cells have stabilized their surface TNF-α receptors. Autocrine TNF-α binds to p55 and a death signal is transmitted to the nucleus. This hypothesis was further supported by the observation that the broad spectrum metalloproteinase inhibitor BB-94 blocked the serum-starvation cell death signal in TIMP-3/DLD cells (data not shown). In this case the BB-94 inhibitor is most likely blocking multiple proteolytic events.

Based upon our and others' findings, a model shown in Figure 6 illustrates how TIMP-3 may protect TNF-α receptors from proteolytic cleavage (shedding) and induce programmed cell death. In this model, DLD carcinoma cells, which do not express endogenous TIMP-3, have active surface MMPs that cleave TNF-α receptors (possibly other ECM factors) releasing the soluble p46 TNF-α inhibitor into the media. The p46 TNF-α inhibitor binds to autocrine TNF-α competing with p55 receptor for ligand. This

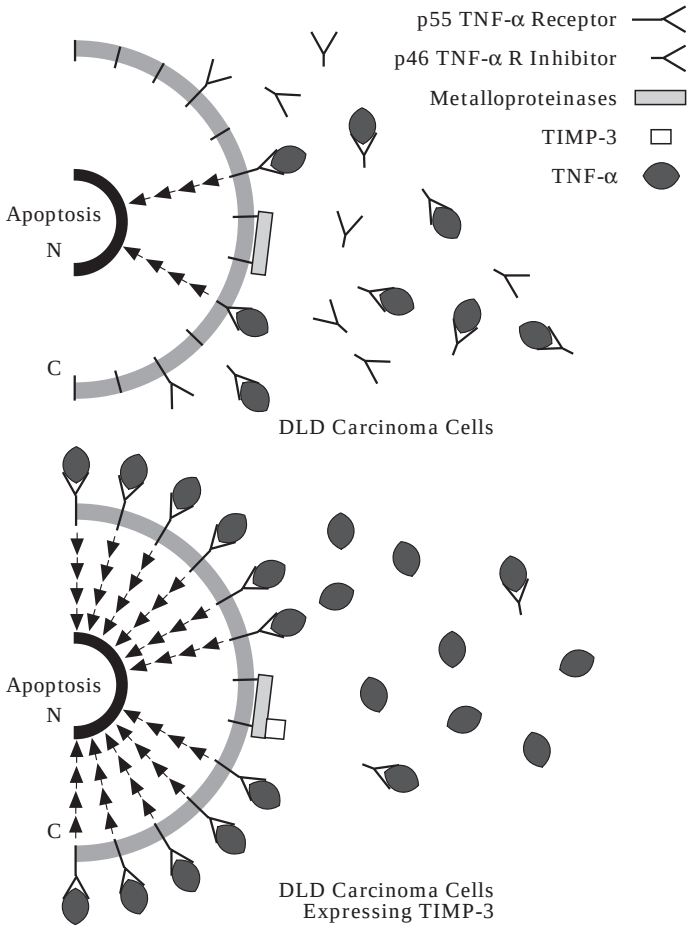

Figure 6. TIMP-3 mediates cell death in human DLD colon carcinoma cells.

DLD cells (vector/DLD, upper panel) do not express TIMP-3 and have active ECM MMPs that cleave p55 TNF-α receptors, releasing the soluble p46 TNF-α inhibitor. Soluble inhibitor binds TNF-α released from the carcinoma cells. Active MMPs cleave the majority of p55 TNF-α receptors, resulting in downregulation of the TNF-α p55 signalling pathway. TIMP-3/DLD cells (lower panel) have inactive (TIMP-3 bound) MMPs, leaving p55 TNF-α receptors available to bind soluble TNF-α released from the carcinoma cell. TNF-α binding to p55 TNF-α receptor transduce a signal through the cytoplasm (C) into the nucleus (N), activating programmed cell death.

competition for TNF-α blocks TNF-α-mediated signalling and explains why conditioned media from vector/DLD and low TIMP-3/DLD cell lines did not kill indicator cells, i.e. the majority of TNF-α receptors are cleaved and inactive, and soluble TNF-α is absorbed by p46. This observation was further supported by the differential sensitivity of the TIMP-3/DLD and vector/DLD cell lines to exogenous TNF-α treatment (Fig. 3). Differential sensitivity to TNF-α most likely occurs because autocrine TNF-α is absorbed by the p46 TNF-α inhibitor that is shed from the vector/DLD cells. The DLD colon carcinoma cells have downregulated TIMP-3 expression (possibly through altered methylation of the gene, 15) and in turn the cell's sensitivity to TNF-α killing, short circuiting the TNF-α signal transduction pathway at the level of the p55 TNF-α receptor (Fig. 6, top). TIMP-3 overexpression in DLD cells inhibits MMP activity, p55 TNF-α receptors are not cleaved, no p46 inhibitor is shed from cell membranes, autocrine TNF-α is active in the media, and liganded p55 TNF-α receptors signal cell death (Fig. 6, bottom).

We have previously shown that TIMP-3 expression in DLD colon carcinoma cells causes complete inhibition of in vivo tumour formation.[16] The findings presented in this report provide an explanation for the mechanism of tumorigenic reversion. TIMP-3 functions as an MMP inhibitor, protects TNF-α receptors from proteolytic cleavage and shedding, and intact p55 TNF-α receptors ensure transduction of cell death signals into the nucleus. Therefore, TIMP-3/DLD cells may undergo cell death in vivo and a non-tumorigenic phenotype results. This observation suggests that TIMP-3, a putative "tumour suppressor gene" can be a critical regulator of transformation. The DLD colon carcinoma gene therapy model demonstrates a pivotal role for TIMP-3 in regulating the integrity of the ECM and during the development of tumours. This model also serves to demonstrate how malignant cells can evade normal mechanisms of immune surveillance, i.e. by downregulating the efficacy of the p55 TNF-α receptor signalling pathway. The subcellular localization of TIMP-3, its profound effects on tumorigenicity, metastasis, and cell cycle regulation, suggest an important role for proper MMP activity during normal cell physiology. Reinforcing the hypothesis that MMPs make logical targets for chemotherapeutic drug development.

MATERIALS AND METHODS

Reagents

Geneticin (G-418), minimum essential medium (MEM), fetal calf serum (FCS), Lipofectin reagent, and isotonic phosphate-buffered saline, pH 7.4 (PBS) were purchased from Life Technologies (Rockville, MD). Propidium iodide (PI), H_2O_2, and 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) were purchased from Sigma Chemical Co. (St Louis, MO). Neutralizing antibodies specific for murine and human TNF-α, anti-human IL-1β antibody, and TNF-α ELISA kit were purchased from Genzyme (Cambridge, MA). Goat anti-human soluble TNF-α receptor I detection antibody (p55-specific) was from R & D Systems (Minneapolis, MN). Biotin-labelled antibody to murine IgG, PE-labelled strepavidin, and peroxide-labelled strepavidin were from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD).

Cell lines

The human DLD-1 colon carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). cDNA coding for TIMP-3 regulated by the CMV promoter (pcDNA3 vector with G418 resistance gene, Invitrogen, San Diego, CA) was transfected into DLD cells using Lipofectin reagent. Stable transfectants were selected with Geneticin at 600 μg/ml.[16] Cell lines from pcDNA3 neo vector and pcDNA3-TIMP-3 (low TIMP-3/DLD cells expresses approximately 5% of the TIMP-3 protein found in the TIMP-3/DLD cell line) were derived by lipofectin-mediated transfection and maintained in MEM supplemented with 15% FCS and 300 μg/ml Geneticin. TIMP-3 expression levels and stability of the transfectant cell lines were determined by Western blotting.[16]

Cell cycle: mitotic index analysis and flow cytometry

Vector/DLD, low TIMP-3/DLD, and TIMP-3/DLD cell lines were seeded ($1–2 \times 10^5$ cells) on glass coverslips in 35-mm dishes with 15% FCS MEM and grown to 60–70% confluence. The cultures were washed with PBS, fixed in cold methanol/acetone, and stained with DAPI (200 ng/ml) in PBS. Individual cells were randomly scored for cell cycle stage, i.e., interphase, prophase, metaphase, anaphase, or telophase.

For FACS analysis the cell lines were seeded (10^6) in 150-mm dishes and grown in 15% FCS MEM to confluence. Fresh media supplemented with 15% or 0.5% FCS was applied and the cultures were incubated at 37°C for 48 h. In some experiments the cultures were harvested during log phase growth at 50–60% confluence. Cells were removed from the dishes either by treatment with trypsin, scraping in cold Tris-buffered saline, pH 7.4, or with enzyme-free cell dissociation solution. They were washed with PBS and suspended in 0.3–0.5 ml of isotonic PBS. For cell cycle FACS analysis, 2 ml of Thornthwaite lysis buffer (25 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 440 μM KH_2PO_4, 335 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM $MgCl_2$, 400 μM $MgSO_4$, 0.2% (W/V) bovine serum albumin, and 0.4% (V/V) NP-40) with 200 μg of RNase A was added at room temperature for 15 min, and PI was added to 50 μg/ml. The preparations were incubated at room temperature for 1 h, then placed in the dark at 4°C for 20–40 h before FACS analysis with a Coulter EPICS Profile (Coulter, Miami, FL).

To estimate the number of TNF-α receptors on the cell surface, all manipulations were performed on ice, 6.5×10^5 cells were incubated with 5 μg of goat anti-human soluble TNF-α receptor I antibody in 100 μl buffer (1% FCS in

PBS) for 30–45 min. The cells were washed in buffer, centrifuged at 800 × ***g*** for 5 min, suspended in 100 µl buffer with 1.5 µg of biotin-labelled rabbit anti-goat IgG for 30–45 min, washed with buffer, and centrifuged at 800 × ***g*** for 5 min. Cells were suspended in 100 µl buffer with 100 ng of PE-labelled streptavidin for 15 min, washed with buffer, centrifuged, and fixed by adding 0.5 ml of fresh 1% paraformaldehyde/PBS (V/V). Cell sorting was preformed with a Coulter EPICS Profile.

Cytotoxicity assays

Cell lines were seeded (5×10^5) on glass coverslips in 35-mm dishes and grown to confluence. The proper medium exchange was made (15% or 0.5% FCS MEM and in some cases 20 µM H_2O_2 or various concentrations of TNF-α were added) and the cultures were incubated at 37°C for various times. In some experiments, neutralizing antibodies were added at the time of medium exchange. Cell death was assessed by staining live cells with PI (1 µM) in isotonic PBS, and scoring percent cells positive for PI uptake with an ultraviolet Leitz laborluz 12 microscope.

DNA isolation and gel electrophoresis assays for DNA damage

Vector/DLD, low TIMP-3/DLD, and TIMP-3/DLD cells (10^6) were seeded into 150-mm dishes and grown in 15% FCS supplemented MEM until confluent. Fresh media, 15% or 0.5% FCS, was applied and the cultures were incubated at 37°C for various times. Cells were removed from the plastic with trypsin, washed in PBS, suspended in 10 vol proteinase K lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.4% SDS, and 10 mg/ml proteinase K), and incubated at 55°C for 45 min and 37°C for 2–3 h. The cell lysate was extracted twice with an equal vol of 1:1 Tris-buffered phenol:chloroform, and precipitated with ethanol. The DNA was suspended in TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA), treated with RNase A for 30 min at 37°C, and extracted with 1:1 Tris-buffered phenol:chloroform and again with chloroform. The DNA was precipitated with 95% ethanol, washed in 80% ethanol, suspended in TE buffer, and the concentration was estimated by optical density measurement. DNA (8–10 µg) was loaded on 1.4% agarose minigels, run at 70 volts for 10 min to 1.5 h, stained with ethidium bromide, and photographed.

Western blotting

Cells (5×10^5) were seeded in 100 mm dishes, grown to confluence, and the appropriate medium (15% or 0.5% FCS) was applied for a 48-h incubation. The cultures were washed with PBS and the cells were removed from the plastic dishes with enzyme-free cell dissociation solution. The cells were washed with PBS and disrupted in eukaryotic cell lysis buffer [10 mM Tris–HCl, pH 7.4, 120 mM NaCl, 10 mM $MgCl_2$, 0.02% NP-40, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml leupeptin]. Protein concentration was estimated with BioRad protein reagent. Conditioned media was concentrated 10× by centrifugation in an Amicon centricon-10 microconcentrator. Approximately 10 µg of protein was run per lane on 10% polyacrylamide gels and the resolved proteins were transferred to nitrocellulose. Equivalent protein loading was monitored by staining the immunoblots with Ponceau S solution before blocking. The blot was incubated with 2 µg/ml of goat anti-human soluble TNF-α receptor I antibody for 1 h, washed with PBS/Tween-20 (0.05%), incubated with 2 µg/ml of secondary biotin-labelled rabbit anti-goat IgG for 1 h, washed extensively, and incubated with 35 ng/ml of peroxidase-labelled streptavidin for 15 min. The blot was washed and TNF-α receptor-specific bands were visualized by ECL (Amersham, Buckinghamshire, UK) exposing on Kodak X-OMAT AR film.

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