Effects of Clusterin Overexpression on TNFα- and TGFβ-Mediated Death of L929 Cells†

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ABSTRACT: Clusterin is a widely distributed and highly conserved protein for which many functions have been proposed. We used transfected L929 cells to study the effect of clusterin expression on the regulation of cell death signals. We showed that high levels of clusterin expression, about 0.2 pg clusterin secreted per cell per 48 h period, specifically protected L929 cells from TNFα-mediated cytotoxicity, while low expression (about 4 fg/cell/48 h) had no effect. However, clusterin expression did not provide transfected L929 cells with protection against death mediated by colchicine, staurosporine or azide. High level expression of clusterin in transfected L929 cells also potentiated the cytotoxicity of TGFβ. It had previously been shown that exposure of L929 cells to TGFβ provides protection against TNFα. We showed that this protective effect is not additive to that of clusterin expression. One interpretation of this data is that it suggests that clusterin and TGFβ may act via a common mechanism to provide protection against the cytotoxicity of TNFα. Our results indicate that an intracellular action of clusterin protein is responsible for protection against TNFα cytotoxicity. Exposure to TNFα induces an increase in the level of cell-associated clusterin and specifically in the level of a novel clusterin molecule, which when analyzed under reducing conditions by SDS/PAGE and immunoblotting appears as two closely spaced bands at about 36 and 38.5 kDa. When analyzed under the same conditions, the normal form of intracellular clusterin, which is present with or without exposure to TNFα, appears as two poorly resolved bands at about 43−45 kDa. Since the novel form of clusterin is also expressed in cells exposed to TGFβ, colchicine, staurosporine, and azide, it may result from toxin-induced disruption of processes of normal cellular protein production.

Clusterin is a protein with an interesting history. It was first described as the major secreted product of ram Sertoli cells, which under certain conditions was capable of enhancing the "clustering" of various cell types (Blaschuk et al., 1983). The secreted form of clusterin appears to interact specifically with a number of biological molecules, including complement components C7, C8, and C9 (Tschopp et al., 1993), apolipoprotein A-I (Jenne et al., 1991), immunoglobulins (Wilson & Easterbrook-Smith, 1992, Wilson et al., 1991), Alzheimer's amyloid β peptide (Ghiso et al., 1993), and SIC, a protein secreted by *Streptococcus pyogenes* (Akesson et al., 1996). Clusterin also binds to the surface of clinical isolates of *Staphylococcus aureus* (Partridge et al., 1996). The functions that have been proposed for clusterin are similarly diverse and include roles in cell aggregation, regulation of the complement system, lipid transport, and protecting cells from apoptosis. However, none of these putative roles has yet been established as genuine physiological functions.

In 1986, the rat homologue of the protein (known as TRPM-2) was reported as being upregulated in the ventral rat prostate during tissue regression following androgen ablation (Montpetit et al., 1986). This system is a model of apoptotic cell death, and the initial report of an association between clusterin expression and apoptosis triggered a rapid expansion in the number of research groups studying clusterin (Jenne & Tschopp, 1992). The ensuing work has shown that clusterin mRNA is expressed in almost all mammalian tissues tested. It is now clear that clusterin expression and apoptosis are not always correlated and in many cases clusterin expression is upregulated in instances of cell injury unrelated to apoptosis. An emerging theme, therefore, is that clusterin is a "stress"-related protein (Rosenberg et al., 1993; Fritz & Murphy, 1993). Analysis of the biosynthesis of clusterin in the HepG2 (Burkey et al., 1991) and MDCK (Urban et al., 1987) cell lines has shown that clusterin is generally secreted from the cell. However, recently it has been shown that in HepG2 and CCL64 cell lines treated with TGFβ, which induces apoptosis of CCL64 cells, a truncated form of clusterin is localized in the nucleus (Reddy et al., 1996a). Furthermore, it has been reported that expression of clusterin in transfected LNCaP cells can protect them from TNFα-induced apoptosis (Sensibar et al., 1995). These data suggested that under conditions of cellular stress clusterin may regulate cell death signals through a mechanism involving the translocation of truncated clusterin to the nucleus.

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The signal transduction pathways of TNFα and TGFβ are incompletely understood. Many cell types express two cell surface TNFα receptors, p55 and p75. The p55 receptor has been clearly implicated in signaling cell death, while the evidence for a similar function for p75 is more controversial. A serine kinase activity and a number of phosphoproteins have been identified associated with TNFα-p55 complexes in U937 cells (Ware et al., 1996), suggesting that phosphorylation events may be important in TNFα signal transduction. Phosphorylation events have also been implicated in the signal transduction of TGFβ (Kekow & Wiedemann, 1995). Furthermore, in L929 cells, the production of reactive oxygen species (ROS) has also been implicated in the mechanism of toxicity of TNFα (Schulze-Osthoff et al., 1994).

Clusterin has recently been identified as binding with the intracellular domains of TGFβ receptors type I and II (Reddy et al., 1996b). We were intrigued by this and by the independent demonstration that in L929 cells TGFβ induces resistance to TNFα-mediated cytotoxicity (Chang, 1995). We wondered whether expression of clusterin in transfected cells might result in a form of the protein which could interact with the intracellular domains of TGFβ receptors (types I and/or II) and thereby confer on the cells resistance to TNFα. To begin an examination of this hypothesis we developed a series of stably transfected L929 cell lines that express human clusterin and examined the effects of TNFα, TGFβ, and several other cytotoxic agents on the survival of these cells and on the biosynthesis and subcellular localization of clusterin.

EXPERIMENTAL PROCEDURES

Materials. Polyacrylamide gel reagents, propidium iodide (PI), 7-aminoactinomycin D (7-AAD), isopropyl β-D-thioglucoside (IPTG), and TGFβ were obtained from Sigma (St. Louis, MO). Nitrocellulose was obtained from Sartorius AG (Göttingen, Germany). Pre-stained molecular weight standards were obtained from Novex Australia Pty Ltd (Sydney, Australia). ECL detection kits and Hyperfilm-ECL for immunoblots were obtained from Amersham (Sydney, Australia). ELISA trays were obtained from Disposable Products (Adelaide, Australia). Human clusterin was purified from serum by immunoaffinity chromatography as described previously (Wilson & Easterbrook-Smith, 1992). Recombinant human TNFα was a gift from Dr. D. Rathgen (Peptide Technology Ltd, Sydney, Australia). The LacSwitch inducible mammalian expression system was purchased from Stratagene (La Jolla, CA) and the pRc/CMV plasmid was purchased from Invitrogen (San Diego, CA). Plasmid purification kits were purchased from Qiagen Inc. (Chatsworth, CA) and restriction enzymes and hygromycin were purchased from Boehringer Mannheim (Sydney, Australia). DMEM:F-12 tissue culture medium and geneticin was purchased from Gibco BRL (Melbourne, Australia). Fetal bovine serum (FBS) and all other tissue culture reagents were obtained from Trace Biosciences Pty Ltd (Sydney, Australia). General chemicals were purchased from Sigma or Ajax Chemicals (Sydney, Australia).

Antibodies. The cell line secreting the $IgG_1\kappa$ anti-clusterin MAb G7 was a gift from Dr. B. Murphy (St Vincents Hospital, Melbourne, Australia). 78E, an $IgG_1\kappa$ anti-clusterin MAb, and DNP-9, an $IgG_1\kappa$ MAb that binds specifically to the 2,4-dinitrophenyl group, have been described before (Wilson & Easterbrook-Smith, 1993). 41D, another $IgG_1\kappa$ anti-clusterin MAb was made by conventional methods using immunoaffinity purified human serum clusterin as antigen. None of the MAbs G7, 78E, and 41D recognized murine clusterin. Rabbit-anti-lac repressor antibody was obtained from Stratagene (La Jolla, CA). HRP-conjugated and FITC-conjugated secondary antibodies were obtained from Silenus Laboratories (Melbourne, Australia).

Molecular Biology. Human clusterin cDNA was obtained as described previously (Wong et al., 1994). Cells were transfected by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). The LacSwitch system containing full-length clusterin cDNA was electroporated into L929 cells, and transfected cells were grown in the presence of both hygromycin (0.4 mg/mL) and geneticin (0.4 mg/mL). This system produced stably transfected cell lines that secrete clusterin after induction with IPTG. Transfected cells resistant to hygromycin and geneticin were screened by dot blot and then immunoblotting for inducible secretion of human clusterin.

Full-length human clusterin cDNA was also ligated into the pRc/CMV plasmid and used to transfect L929 cells as above. In this case initial selection was by growth in 0.4 mg/mL geneticin. Transfected cells resistant to geneticin were screened by dot blot and then immunoblotting for constituitive secretion of human clusterin. As a control, we also transfected L929 cells with the pRc/CMV plasmid lacking an insert. In this case, transfectants resistant to geneticin were selected at random. Once selected, all L929 transfectants were cloned twice by limiting dilution and, where appropriate, rescreened for expression of clusterin.

Cell Culture. The L929 (murine fibrosarcoma) cell line was obtained from the ATCC (Rockville, MD). Cultured L929 cells were grown in DMEM:F-12 tissue culture medium containing 10% (v/v) FBS and hygromycin and/or geneticin as appropriate. For experiments testing the cytotoxicity of various agents, equal numbers of transfected L929 cells were introduced into the wells of 24-well tissue culture plates and incubated for at least 16 h before treatment was started. After treatment, cells were removed from wells by incubation for 5 min at 37 °C with 0.05% (w/v) trypsin, 0.02% (w/v) EDTA (trypsin/EDTA). When testing for the effects of secreted proteins adsorbed to the culture vessel surface on cell survival, or the expression of clusterin at the cell surface, adherent cells were removed from the plastic surface by removing the culture medium and overlaying the cells with 0.5× PBS/EDTA (68.5 mM NaCl, 1.3 mM KCl, 0.74 mM KH_2PO_4, 4.0 mM Na_2HPO_4, 5 mM EDTA, pH 7.4). After 5−10 min incubation, the cells were removed by vigorous washing with 0.5× PBS/EDTA.

Preparation of Cell Lysates, Fluoroimaging, and Dot Blot Assays. To prepare cytoplasmic fractions (i.e. cytosol plus ER/Golgi), cells were lysed in TTX buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.2% Triton X-100) on ice for 15 min. The lysates were centrifuged at 10 000 rpm in a microfuge for 5 min, and the supernatants were mixed 1:1 with sample buffer [SB; 0.5 M Tris-HCl, 20% (v/v) glycerol, 5% (w/v) sodium dodecyl sulfate, 0.005% (w/v) bromophenol blue, pH 6.8] and electrophoresed on 10% SDS polyacrylamide gels. Nuclear extracts were made as described in (Reddy et al., 1996a); briefly, cell were lysed as above and the total lysate was overlayed on a cushion of 10% (w/v) sucrose in TTX buffer before centrifuging at 800*g* for 10 min. The pelleted nuclei were resuspended in SB and boiled

for 5 min before loading on to 10% SDS polyacrylamide gels and electrophoresis. Total cell extracts were prepared by washing cells once in PBS, and then lysing them directly in SB and boiling them for 10 min before loading on to SDS polyacrylamide gels. Samples separated by SDS/PAGE were electrophoretically transferred to nitrocellulose membrane (Sartorius, Melbourne, Australia) using a semi-dry Bio-Rad Trans-Blot SD apparatus (Bio-Rad, Hercules, CA). Fluoroimaging was performed using a Storm 840 fluoroimager (Molecular Dynamics, Sunnyvale, CA), and all data analysis was carried out using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The amount of clusterin secreted by transfected cells was quantified by dot blot assay using different amounts of purified human serum clusterin added to cell culture medium to construct a standard curve. The cells were incubated in culture medium for 48 h, and aliquots of the culture supernatant and standards then spotted onto a nitrocellulose membrane. Bound clusterin was detected with MAb G7 followed by a sheep-anti-mouse IgG-HRP conjugate and then ECL. A GS670 densitometer (Bio-Rad, Hercules, CA) and Molecular Analyst software (Bio-Rad, Hercules, CA) were used to capture images of ECL-exposed photographic film and quantitate the results of dot blot experiments.

Flow Cytometry. Flow cytometry data were collected using a Becton Dickinson FACSort and analyzed using CELLQuest software (Becton Dickinson, Sydney, Australia). Annexin V-FLUOR (FITC conjugate) was obtained from Boehringer Mannheim (Sydney, Australia) and used following the manufacturer's instructions. The red fluorescing nuclear stain 7-AAD was used to discriminate between dead, apoptotic and viable cells, as described in (Schmid et al., 1994). There was no difference in the pattern of 7-AAD staining of adherent cells removed from the culture surface with trypsin/EDTA versus 0.5× PBS/EDTA (data not shown). In one type of experiment it was necessary to establish the level of intracellular clusterin for live versus dead cells in the same culture. This was done in the following way. For each test, 2.5×10^6 cells were pelleted by centrifugation, resuspended in 50 μL of 20 μg/mL 7-AAD in PBS, and incubated on ice for 20 min. The cells were then washed 3× with cold PBS and permeabilized by resuspension in 1 mL of ice-cold 50% (v/v) ethanol in PBS, followed by vortexing for 1 min and incubation on ice for a further 4 min. The cells were then washed 3× with cold PBS before incubating them with G7 MAb or a control MAb, followed by a sheep-anti-mouse Ig-FITC (SaMIg-FITC) conjugate. Dead cells were discriminated by their high red fluorescence, while cells that in culture still had an intact cell membrane were discriminated by their relatively low red fluorescence. For each flow-cytometric analysis, data were acquired from 10 000 cells.

RESULTS

Production of Stably Transfected Cell Lines

L929-LS.clus, a stably transfected L929 cell line containing clusterin cDNA, inducibly secretes human clusterin when IPTG is added to the culture medium. When analyzed by SDS/PAGE under reducing and non-reducing conditions, human clusterin secreted by L929-LS.clus behaved similarly to clusterin immunoaffinity purified from human serum (Figure 1A). The results of dot blot experiments (not shown) indicated that over a period of 48 h, IPTG-induced L929-LS.clus cells secreted about 4 fg of clusterin per cell. L929-pRc.clus cells were made by stably transfecting L929 cells with the pRc/CMV plasmid containing full-length clusterin cDNA. These cells constitutively secrete human clusterin. When analyzed by SDS/PAGE under reducing and non-reducing conditions, human clusterin secreted by L929-pRc.clus cells behaved similarly to clusterin immunoaffinity purified from human serum (Figure 1B). L929-pRc.clus cells secreted about 0.2 pg of clusterin per cell over 48 h (estimated from dot blot experiments, not shown). Therefore, L929-pRc.clus cells secrete human clusterin at a rate about 50 times that of IPTG-induced L929-LS.clus cells. L929-pRc cells were made by stably transfecting L929 cells with the pRc/CMV plasmid lacking an insert. These cells were used as controls, and do not express human clusterin.

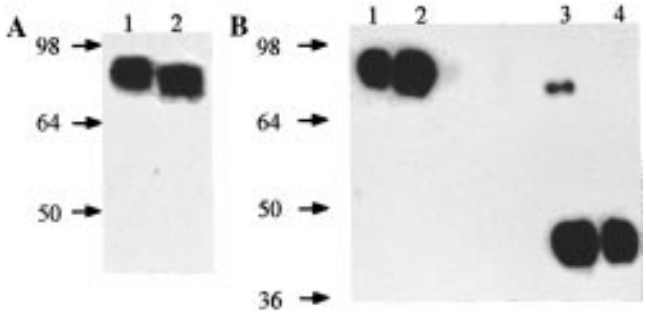

FIGURE 1: Immunoblots showing clusterin protein secreted by induced L929-LS.clus cells (A) and L929-pRc.clus cells (B). (A) L929-LS.clus cells were grown for 24 h in 5 mL of culture medium in a 25 cm^2 tissue culture flask in the presence of 5 mM IPTG. About 8×10^6 cells were recovered from the flask after the 24 h period. A 20 μL aliquot of culture supernatant was loaded onto a 10% SDS/PAGE gel (lane 2). Purified human serum clusterin (200 ng) was loaded into lane 1. The samples were electrophoresed under non-reducing conditions (results for reducing conditions not shown). After completion of gel electrophoresis, separated proteins were electrophoretically transferred to a nitrocellulose membrane and probed with MAb G7. The position of bound MAb was detected with a sheep-anti-mouse Ig-HRP conjugate followed by ECL. (B) L929-pRc.clus cells were grown for 24 h in 5 mL of culture medium in a 25 cm^2 flask and then 25 μL of culture supernatant loaded onto lanes 2 and 4. About 3×10^6 cells were recovered from the flask after the 24 h period. Purified human serum clusterin (200 ng) was loaded into lanes 1 and 3. Electrophoresis was performed under either non-reducing (lanes 1 and 2) or reducing conditions (lanes 3 and 4). After completion of gel electrophoresis, separated proteins were electrophoretically transferred to a nitrocellulose membrane and probed with a cocktail of the MAbs G7, 78E, and 41D. The position of bound MAb was detected with a sheep-anti-mouse Ig-HRP conjugate followed by ECF and fluoro-imaging. No clusterin was detectable in supernatants of L929-pRc cells (not shown). The results shown are representative of at least three independent experiments.

Mode of TNFα-Induced Death in Transfected L929 Cells

Having demonstrated that the transfected cells produced clusterin with similar structural properties to clusterin purified from human serum, we sought to establish the effects of clusterin production on the susceptibility of the cells to TNFα. Others have shown that exposure of L929 cells to TNFα can elicit either necrotic (Schulze-Osthoff, 1994; Schulze-Osthoff et al., 1994) or apoptotic (Barbiero et al., 1995; Kyprianou, 1991) death. Therefore, we first established which of these modes of death was induced in our cultures by TNFα. Using flow cytometry and fluorescent nuclear stains, it is possible to discriminate between apoptotic, viable, and dead cells. This is now a well-established technique and has been demonstrated for a wide variety of

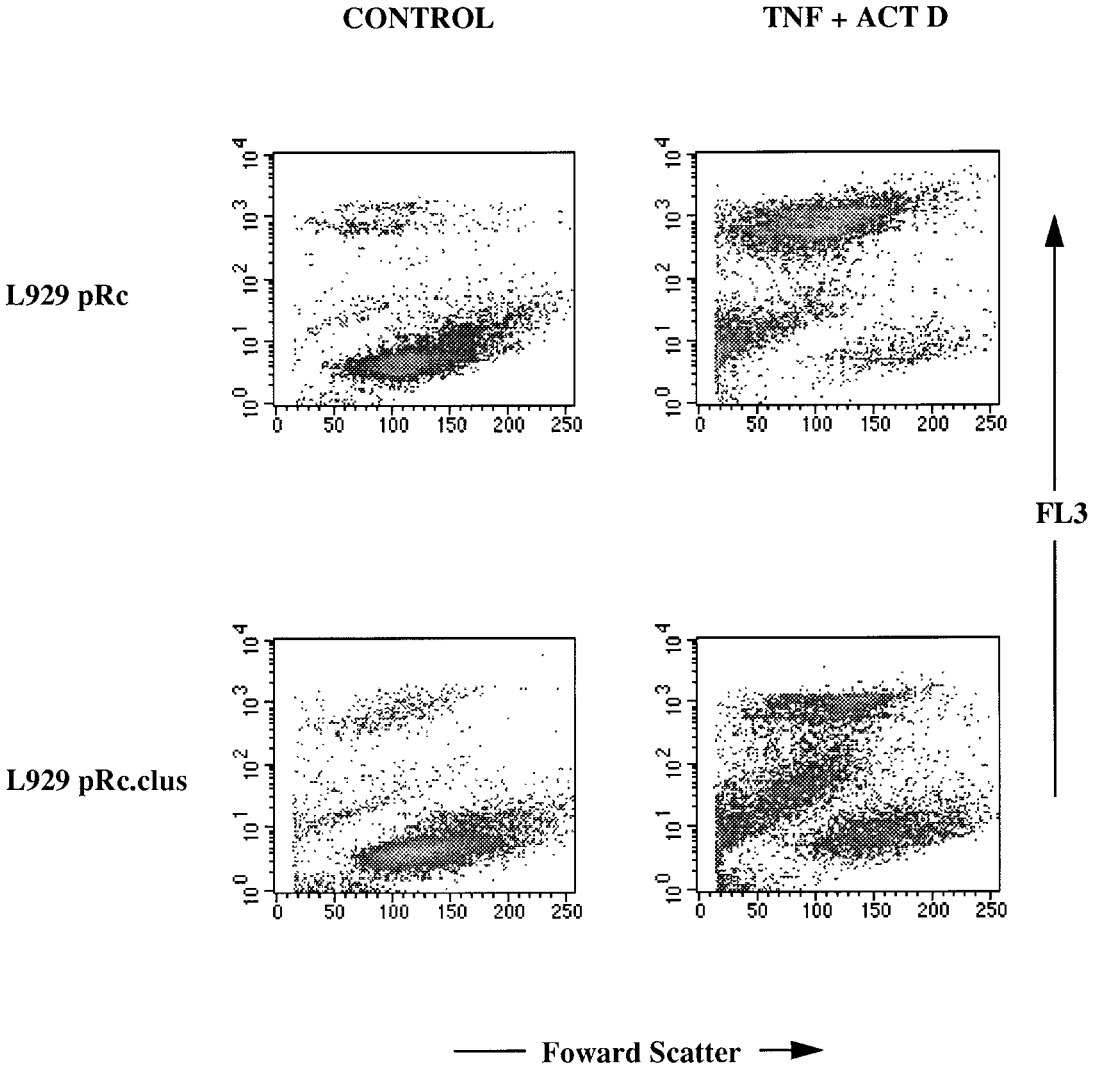

FIGURE 2: Flow cytometry density plots of 7-AAD uptake (FL3) versus foward scatter in L929-pRc and L929-pRc.clus cells exposed to the treatments indicated. The treatments were: untreated control and 100 ng/mL TNFα + 4 μg/mL actinomycin D for 9 h. The results shown are representative of many independent experiments. Results similar to those shown for cells exposed to TNFα + actinomycin D were obtained for cells treated with 100 ng/mL TNFα for 24 h (not shown).

cell types and for each of the nuclear stains Hoechst 33342 (Dive et al., 1992), 7-AAD (Schmid et al., 1994), propidium iodide (Zamai et al., 1996), YOPRO-1 (Idziorek et al., 1995) and ethidium bromide (Lyons, 1992). This ability has been ascribed to differences in cell membrane permeability: dead cells stain the most brightly, lacking an intact cell membrane to exclude the dye; apoptotic cells stain with a lower intermediate intensity, their membranes having an intermediate permeability; and viable cells stain the least brightly, having the lowest membrane permeability.

We showed that TNFα induced changes indicative of both apoptotic and necrotic death in L929 cultures. Exposure to TNFα or TNFα plus actinomycin D induced cell shrinkage and the appearance of a population of cells with intermediate fluorescence when stained with 7-AAD (Figure 2). It is also clear from the results shown in Figure 2 that, following treatment with TNFα and actinomycin D, a greater proportion of L929-pRc.clus cells remained viable (i.e. showed low staining with 7-AAD) when compared with L929-pRc cells (see below). Following treatment with TNFα, the characteristics of cell shrinkage and intermediate staining with 7-AAD were always more apparent in L929-pRc.clus cells than in control L929-pRc cells (e.g., data shown in Figure 2). However, when the same cells were stained with acridine orange and examined by fluorescence microscopy, no nuclear condensation or fragmentation was seen (data not shown). Following staining with PI (Nicoletti, 1991), flow-cytometric analysis of the DNA content of L929 cells exposed to TNFα did not show a discrete "hypodiploid" peak, typical of apoptotic cells, but rather showed cells with a broad range of sub-diploid DNA content (Figure 3). Furthermore, analysis of DNA from cells exposed to TNFα by agarose gel electrophoresis showed visible DNA degradation but no evidence of oligonucleosomal cleavage (i.e. no "DNA ladders"; data not shown). Thus, when exposed to TNFα, transfected L929 cells showed changes in their nuclei and DNA content expected of necrotic death (Kerr, 1995). We also tested whether L929 cells treated with TNFα exposed phosphatidylserine at the cell surface, a change normally associated with apoptotic cells. We found that annexin V-FITC bound to the surface of L929 cells treated with a variety of agents, including azide, staurosporine, colchicine and, to a lesser extent, TNFα, but not to the surface of untreated L929 cells (data not shown).

In summary, TNFα-treated transfected L929 cells showed a reduction in cell size, intermediate staining with 7-AAD, and a measurable exposure of PS at the cell surface, features normally associated with apoptotic cells. However, they did not demonstrate either of two other characteristics normally found associated with apoptotic cells—nuclear condensation

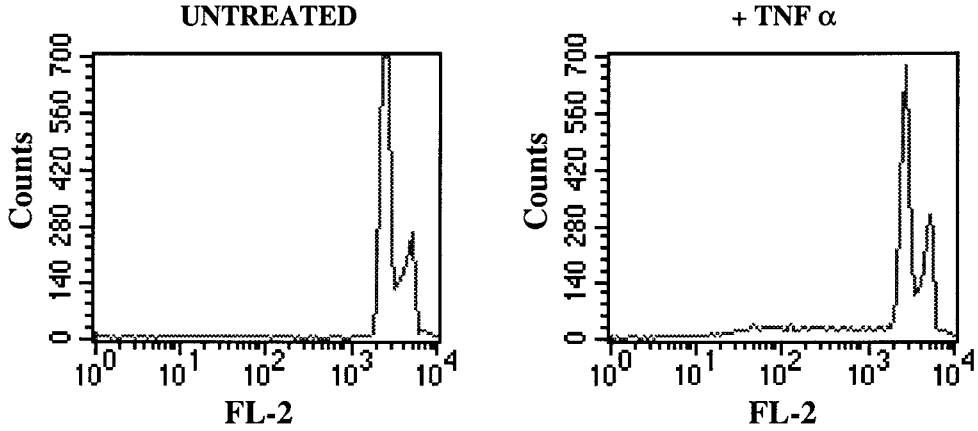

FIGURE 3: Flow cytometry histograms showing the orange fluorescence (FL2) of permeabilized L929-pRc.clus cells stained for DNA content with propidium iodide (PI), using the method of (Nicoletti et al., 1991). Cells were either untreated or exposed for 24 h to 100 ng/mL TNFα (indicated) before staining with PI. The results shown are representative of more than three independent experiments. When treated with TNFα or TNFα plus actinomycin D, wild type L929 and L929-pRc cultures also showed cells with a broad range of levels of sub-diploid DNA content (not shown).

and fragmentation, or internucleosomal DNA "laddering". The finding of features of both necrosis and apoptosis in the death of a single cell type is not unique. Similar duality in the nature of death has been reported for other fibroblastic cell types (Simm et al., 1997), and specifically in response to TNFα (Laster et al., 1988). This may, in part, explain the apparent contradiction between earlier reports claiming that TNFα induces apoptosis (Barbiero et al., 1995; Kyprianou, 1991) versus necrosis (Schulze-Osthoff, 1994; Schulze-Osthoff et al., 1994) in L929 cells. In our results, the 7-AAD-intermediate population represents cells with an intact but "leaky" plasma membrane. These cells cannot be regarded as genuinely apoptotic, although they clearly represent a stage of TNFα-mediated death in this system.

We next used 7-AAD to measure the extent of death in transfected L929 cells exposed to a number of toxic agents. In subsequent work, cells showing "high" staining with 7-AAD were defined as "dead", while cells showing "low" staining with 7-AAD were defined as "viable". Another common method of discriminating live from dead cells is to briefly stain cells with a low concentration of the fluorescent nuclear dye PI immediately prior to flow-cytometric analysis. In a number of independent experiments, the proportion of L929 cells defined as dead by staining with 7-AAD matched that defined as dead by staining with PI (data not shown).

Overexpression of Clusterin Protects L929 Cells from the Cytotoxicity of TNFα but Not Other Agents

We tested the effects of TNFα, and actinomycin D in combination with TNFα, on the survival of transfected L929 cells. Actinomycin D has been reported to enhance the cytotoxicity of TNFα in L929 cells (Kyprianou, 1991), an effect that was confirmed by our results. When L929-pRc.clus cells were exposed to TNFα or TNFα plus actinomycin D, they showed significantly less cell death than control L929-pRc cells (Figure 4A). In addition, we showed that under the same conditions the extent of DNA fragmentation in L929-pRc.clus cells was significantly less than that in L929-pRc cells (Figure 4B). These results indicate that the level of clusterin expressed by L929-pRc.clus cells specifically protects them from the toxicity of TNFα. This protective effect was dose-dependent because our results indicate that the level of clusterin expression by induced L929-LS.clus cells, which is about 50 times less than that of L929-pRc.clus cells, was insufficient to produce any effects on the toxicity of TNFα, as judged by the extent of either cell death (Figure 4C) or DNA fragmentation (data not shown).

Colchicine, an agent that potently disrupts microtubule structure, and staurosporine, an inhibitor of protein kinases, have both been reported to induce apoptosis in a variety of cell types (Bertrand et al., 1994; Bumbasirevic et al., 1995; Tsukidate et al., 1993). We tested the effects of colchicine, staurosporine, and azide (a well-known cytotoxic agent that acts as a respiratory poison) on the survival of transfected L929 cells. For L929 cultures exposed to staurosporine or colchicine, we observed condensed and/or fragmented nuclei in acridine orange stained cells, and demonstrated by flow cytometry "intermediate" staining of cells with 7-AAD (Schmid et al., 1994) and a discrete hypodiploid peak in permeabilised cells stained for cellular DNA content with propidium iodide (Nicoletti, 1991). These data (not shown) indicate that staurosporine and colchicine both induce apoptosis in L929 cells. Similar analyses indicated that azide induced primarily necrotic death in L929 cells.[1] There was no difference in the survival of L929-pRc versus L929-pRc.clus cells exposed to colchicine, staurosporine, or azide (Figure 5). This implies that although overexpression of clusterin provides protection against the cytotoxicity of TNFα (shown in Figures 4A and B), it does not provide a general protective effect against noxious agents.

Overexpression of Clusterin Affects the Sensitivity of L929 Cells to TGFβ and TNFα

It has been previously reported that exposure of L929 cells to TGFβ provides them with protection against TNFα-mediated cytotoxicity (Chang, 1995). We were interested to see if the expression of clusterin in transfected cells would influence the effects of TGFβ on TNFα-mediated cell death. We confirmed that pre-exposure of L929.pRc cells to TGFβ does protect them from death induced by subsequent exposure to TNFα (Figure 6). In contrast, pre-exposure of L929-pRc.clus cells to TGFβ produced no effect on the sensitivity of the cells to TNFα (Figure 6). These results

[1] D. Humphreys, unpublished observations.

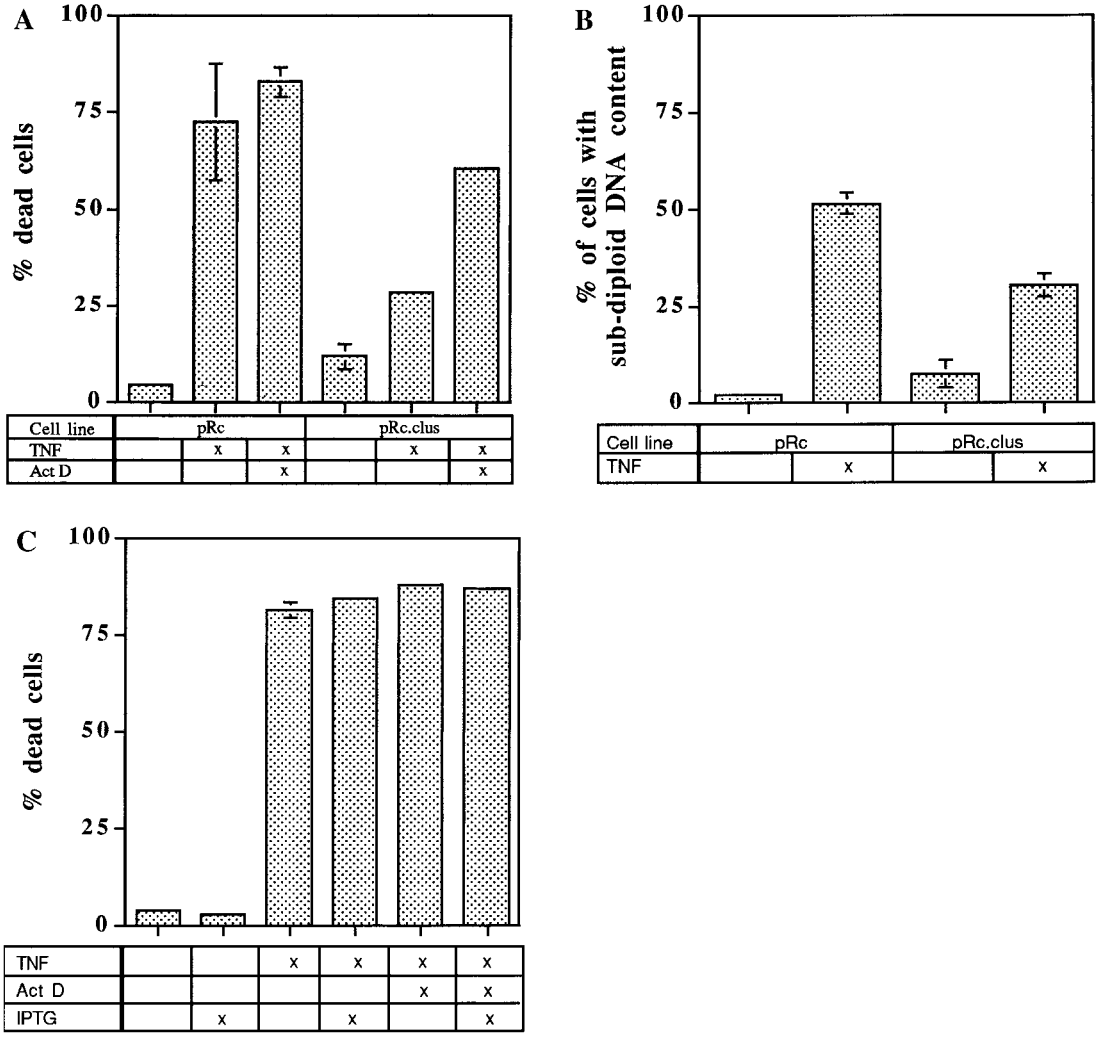

FIGURE 4: (A) Histograms showing the percentage of L929-pRc (pRc) and L929-pRc.clus (pRc.clus) cells defined by 7-AAD staining and flow-cytometric analysis as dead in untreated control cultures or cultures exposed to 100 ng/mL TNFα for 24 h or to 100 ng/mL TNFα + 4 μg/mL actinomycin D for 9 h. Relative to the control pRc cells, pRc.clus cells showed significantly less cell death in response to TNFα or TNFα + actinomycin D ($p < 0.05$, Student's *t*-test). (B) Histograms showing the percentages of cells with hypodiploid DNA content for cultures of L929-pRc and L929-pRc.clus cells treated with 100 ng/mL TNFα for 24 h, or untreated control cultures. To determine DNA content, cells were stained with PI as described in (Nicoletti et al., 1991), and the percentage of cells with different levels of DNA determined using CELLQuest software. Following exposure to TNFα, there is significantly less DNA fragmentation in pRc.clus cells versus L929-pRc cells ($p < 0.01$, Student's *t*-test). (C) Histograms showing the percentages of uninduced or IPTG-induced L929-LS.clus cells defined by 7-AAD staining and flow-cytometric analysis as dead in untreated control cultures or cultures exposed to 100 ng/mL TNFα for 24 h or to 100 ng/mL TNFα + 4 μg/mL actinomycin D for 9 h. None of the differences between uninduced and IPTG-induced LS cells are statistically significant ($p > 0.2$, Student's *t*-test). For A−C, the data shown are representative of three independent experiments. In each case, the table below the *x*-axis indicates the conditions applied (indicated by "×"). Each histogram represents the mean of three replicate measurements, and the error bars shown represent SD of the mean. In a number of cases the SD is too small to be visible.

demonstrate that the protective effects of clusterin and TGFβ against the cytotoxicity of TNFα are not additive. Furthermore, L929-pRc.clus cells show significant cell death in response to TGFβ, while L929-pRc cells do not die under the same conditions (Figure 6). This result suggests that in L929 cells high level expression of clusterin potentiates the cytotoxicity of TGFβ.

Secreted or Exogenous Clusterin Does Not Protect Cells from TNFα-Mediated Cytotoxicity

The data in Figure 4 (A, B) show that L929-pRc.clus cells, which express high levels of clusterin, are protected against TNFα-mediated death. To establish the cellular site at which clusterin provides this protective effect, we tested whether clusterin secreted into the culture medium, or added exogenous clusterin, was able to protect cells from TNFα-mediated cytotoxicity. Neither culture supernatant from L929-pRc.clus cells nor exogenous clusterin at 60 μg/mL affected the response of L929-pRc cells to TNFα (Figure 7A,B). Furthermore, there was no significant difference in the effects of TNFα on L929-pRc cells grown on new culture wells or wells in which L929-pRc had previously been grown, compared to wells on which L929-pRc.clus cells had previously been cultured (cells were removed from culture surfaces by treatment with 0.5× PBS/EDTA; data not shown). This result suggests that it is very unlikely that L929-pRc.clus cells can secrete molecules that become bound to the culture vessel surface and provide protection against TNFα-mediated cell death. Lastly, using flow cytometry, we found that there is negligible clusterin associated with the surface of L929-pRc.clus cells (data not shown), suggesting that cell surface-bound clusterin was not producing the effect. In summary, the results indicate that protection against TNFα-mediated cell death is afforded by an intracellular action of clusterin protein.

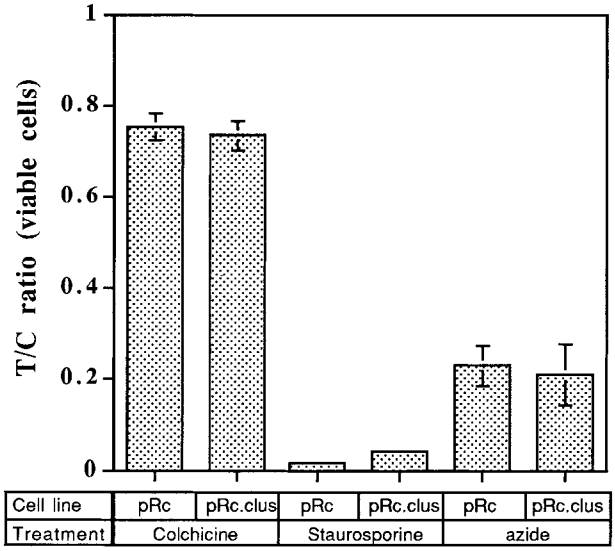

FIGURE 5: Histograms showing the treatment:control ratio (T/C ratio) for the percentage of viable L929-pRc (pRc) and L929-pRc.clus (pRc.clus) cells in cultures exposed to the treatments indicated compared with control untreated cultures. In each case, the table below the *x*-axis indicates the cell line and the conditions applied (indicated by "×"). The treatments were 2 μM colchicine for 48 h, 1 μM staurosporine for 16 h, and 0.6% (w/v) sodium azide for 24 h. Viable cells were determined by 7-AAD staining and flow-cytometric analysis. Each histogram represents the mean of three replicate measurements, and the error bars shown represent SD of the mean. In a number of cases the SD is too small to be visible. The data shown are representative of three independent experiments. None of the differences between L929-pRc and L929-pRc.clus cells are statistically significant ($p > 0.05$, Student's *t*-test).

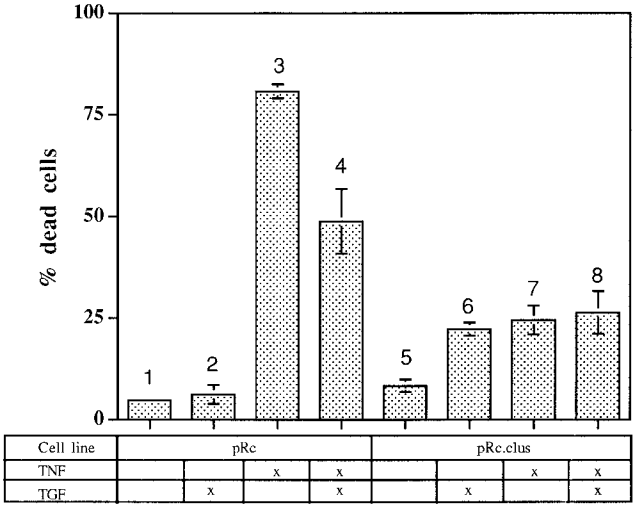

FIGURE 6: Percentages of 7-AAD stained cells defined by flow-cytometric analysis as dead from cultures of L929-pRc (pRc) or L929-pRc.clus (pRc.clus) cells exposed to 2 ng/mL of TGFβ for 48 h and/or 100 ng/mL TNFα for 24 h. Cultures treated with both TGFβ and TNFα first had a 24 h incubation with TGFβ followed by a 24 h incubation with both TGFβ and TNFα. In each case, the table below the *x*-axis indicates the cell line and the conditions applied (indicated by "×"). Each data set represents the mean for triplicate cultures and the error bars shown represent SD of the mean in each case. The result shown is representative of three independent experiments. There was no significant difference in the proportion of dead cells in untreated pRc versus pRc.clus cultures (1 vs 5; Student's *t*-test, $p > 0.5$). Whether exposed to TNFα alone, or to TGFβ followed by TNFα, there was significantly less cell death in pRc.clus versus pRc cells (7 vs 3, $p < 0.001$, and 8 vs 4, $p < 0.05$, respectively; Student's *t*-tests). There were significantly more dead cells in cultures of pRc.clus cells exposed to TGFβ relative to pRc cultures treated the same way (6 vs 2; $p < 0.005$, Student's *t*-test). Following exposure to TNFα, there were significantly more dead cells in control pRc cultures relative to those that had been pre-exposed to TGFβ (3 vs 4; $p < 0.001$, Student's *t*-test). Under the same conditions, there was no significant difference between the level of dead cells in TNFα-treated pRc.clus cultures versus those that had been pre-exposed to TGFβ prior to treatment with TNFα (7 vs 8; $p > 0.2$, Student's *t*-test).

Analysis of Intracellular Forms of Clusterin in Transfected L929 Cells

Analysis of lysates of induced L929-LS.clus and L929-pRc.clus cells by SDS/PAGE and immunoblotting revealed the presence in both cell types of two forms of clusterin with apparent molecular weights, under non-reducing conditions, of about 80 and 64 kDa (Figure 8). The 80 kDa form was also found in cell culture supernatants and dissociated into two subunits under reducing conditions, indicating that it had been appropriately cleaved at the $Asp^{205}-Ser^{206}$ bond (Kirszbaum et al., 1989). The 64 kDa form was not found in cell culture supernatants and did not dissociate into subunits on reduction. We also performed similar analyses of lysates prepared from pRc.clus exposed for 48 h to 100 ng/mL TNFα. Significantly more clusterin was found associated with all cell fractions prepared from TNFα-treated L929-pRc.clus cells versus untreated control cells (Figure 9A). The majority of this increase appeared to be associated with the nuclear fraction. Nuclear-associated clusterin was physically indistinguishable from purified human serum clusterin, which is itself indistinguishable from cell-secreted clusterin (Figures 1B and 9A).

Earlier work had shown that when purified exogenous clusterin was added to nutrient-deprived cultures of various cell types, clusterin bound to the nuclei of dead cells.[2] We therefore suspected that in TNFα-treated cultures of L929-pRc.clus cells, cell-secreted clusterin was simply binding to the nuclei of dead cells once their cell membrane was no longer intact. We tested this hypothesis by first staining L929-pRc.clus cells, either untreated or treated with TNFα, briefly with 7-AAD and then permeabilizing the membranes of the cells before detecting intracellular clusterin with G7 MAb followed by SaMIg-FITC (see Experimental Procedures). Two-color flow-cytometric analysis then allowed us to determine the relative amounts of clusterin associated with dead cells (discriminated as "strongly" stained with 7-AAD) versus cells still possessing an intact cell membrane (discriminated as "weakly" stained with 7-AAD) (Figure 9B). This analysis confirmed that in TNFα-treated L929-pRc.clus cultures, relative to cells with an intact cell membrane, dead cells had greater amounts of cell-associated clusterin (Figure 9B). Inspection of the stained cells by fluorescence microscopy indicated that clusterin was predominantly localized on the nuclei and cell membranes of dead cells (data not shown). We also used flow cytometry to demonstrate that there was no difference in the extent of binding of clusterin−FITC versus bovine serum albumin−FITC to dead cells (discriminated by "high" staining with 7-AAD) in TNFα-treated L929-pRc.clus cultures (data not shown). This indicates that binding of cell-secreted clusterin to the nuclei of dead cells is "artifactual" and unlikely to have any physiological significance.

Our results also demonstrate that, when analyzed under non-reducing conditions, TNFα induces an increase in the 64 kDa form of clusterin in the cytoplasmic fraction of L929-

[2] M. R. Wilson, unpublished observations.

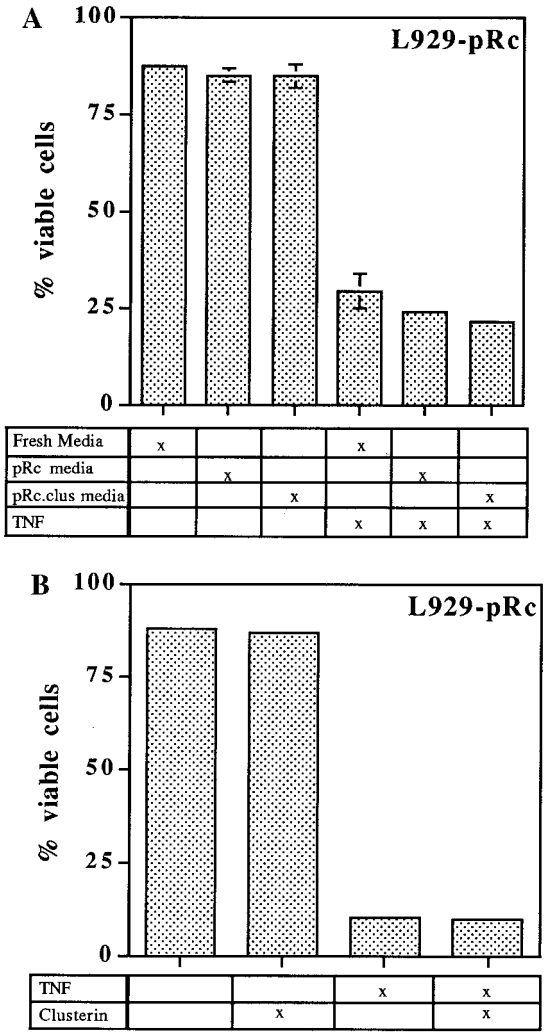

FIGURE 7: Influence of (A) secreted molecules and (B) added exogenous clusterin on the cytotoxic effects of TNFα on L929 cells. Histograms showing the percentages of viable L929-pRc cells, defined by 7-AAD staining and flow-cytometric analysis, from cell cultures exposed to a range of conditions. (A) Where TNFα was applied, pRc cells were exposed for 24 h to 100 ng/mL TNFα added to fresh culture medium or to culture supernatant removed from other cell cultures (which had previously been incubated for 24 h). In each case, the table below the *x*-axis indicates the conditions applied (indicated by "×"). There were no significant differences in the percentages of viable cells in cultures exposed to the different culture media, with or without TNFα (single-factor ANOVA, $0.05 < p < 0.025$). (B) pRc cells were incubated with TNFα with or without the addition of 60 μg/mL of purified exogenous human serum clusterin. In each case, the table below the *x*-axis indicates the conditions applied (indicated by "×"). The addition of exogenous clusterin had no effect on the viability of the cells under any of the conditions tested ($p > 0.2$, Student's *t*-test). Each data set represents the mean for triplicate cultures and the error bars shown represent SD of the mean in each case. In a number of cases the SD is too small to be visible. The results shown are representative of three independent experiments.

pRc.clus cells (Figure 9A). In addition, our results show that in cytoplasmic and total cell fractions prepared from TNFα-treated L929-pRc.clus cells a form of clusterin is detected on immunoblots that is difficult to detect in fractions prepared from untreated L929-pRc.clus cells (Figures 8 and 9C and data not shown). This novel form of clusterin is most clearly resolved under reducing conditions, where it can be seen as a closely spaced pair of bands migrating at apparent sizes of about 36 and 38.5 kDa. In the same preparations, the normal processed form of clusterin is detected as a poorly resolved pair of bands migrating at an

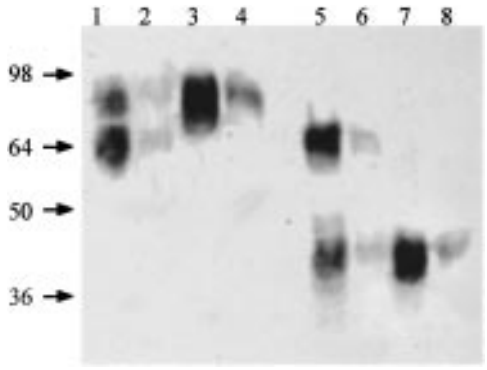

FIGURE 8: Immunoblot of whole cell lysates and tissue culture supernatants from L929-LS.clus cells and L929-pRc.clus cells. L929-LS.clus or L929-pRc.clus cells were grown in 5 mL of culture medium in 25 cm^2 flasks for 24 h. L929-LS.clus cells were grown in the presence of 5 mM IPTG to induce clusterin expression. About 3×10^6 cells were recovered from each flask after the 24 h period. Cells were removed from the flasks with trypsin/EDTA, washed, and then lysed in a volume of 1 mL as described in Experimental Procedures. Aliquots of cell lysates (40 μL) and culture supernatants (20 μL) were electrophoresed on a 10% SDS−polyacrylamide gel and then electrophoretically transferred to nitrocellulose membrane. The membrane was then probed with a cocktail of G7, 78E, and 41D MAbs (which only recognize human clusterin). Bound MAb was detected with a sheep-anti-mouse Ig-HRP conjugate, followed by ECF and fluoroimaging. Lane 1, whole cell lysate from L929-pRc.clus; lane 2, whole cell lysate from induced L929-LS.clus; lane 3, culture supernatant from L929-pRc.clus; lane 4, culture supernatant from induced L929-LS.clus. Lanes 1−4 were run under non-reducing conditions. Lanes 5−8 contain identical samples to those in lanes 1−4 but were run under reducing conditions. The result shown is representative of several independent experiments.

apparent size of about 43−45 kDa (Figure 9C). The 36/38.5 kDa form of clusterin may also be present to a lesser extent in nuclear fractions prepared from TNFα-treated L929-pRc.clus cells, although, by immunoblot analysis, this is difficult to discern because of the large amounts of normal processed clusterin bound to dead cell nuclei. The 36/38.5 kDa clusterin is also detectable in lysates of L929-pRc.clus cells exposed to TGFβ, azide, staurosporine, or colchicine (data not shown). Like the constitutive form of clusterin, the 36/38.5 kDa form has clearly been internally proteolytically cleaved.

DISCUSSION

There is a clear association between increased clusterin expression and a wide variety of instances of cellular "stress". Examples of these include regression of the rat prostate following androgen ablation; the kidney following nephrotoxic injury or damage caused by hydrostatic pressure following ureteral obstruction; and a number of degenerative conditions including Alzheimer's and Pick's diseases, myocardial infarction, and a variety of cancers (Jenne & Tschopp, 1992). It has been suggested that clusterin expression may be a general cellular response to a variety of noxious insults.

Here we report that overexpression of clusterin in L929-pRc.clus cells provides them with resistance to TNFα-mediated cytotoxicity, relative to control L929-pRc cells (Figure 4A and B). Importantly, this protection was specific to TNFα, since L929-pRc and L929-pRc.clus cells were equally susceptible to death mediated by colchicine, staurosporine or azide (Figure 5). Protection against TNFα cytotoxicity was absent in induced L929-LS.clus cells, which express clusterin at a level about 50 times lower than that found in L929-pRc.clus cells (Figure 4C).

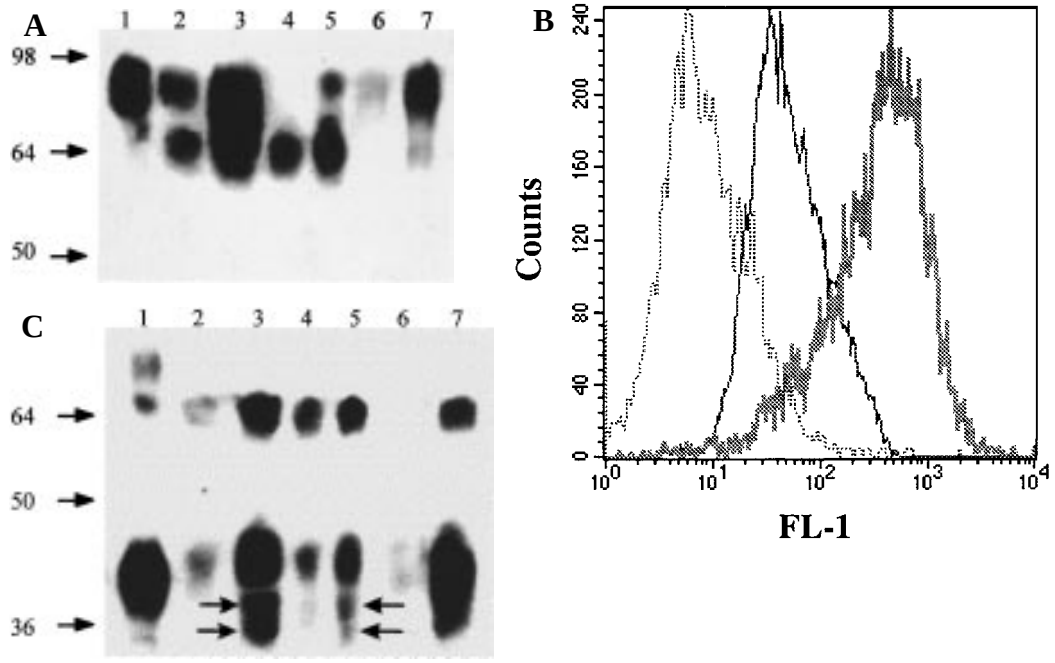

FIGURE 9: (A) Immunoblot of various cell fractions prepared from L929-pRc.clus cells that had been exposed to 100 ng/mL TNFα for 48 h, or untreated controls. Cells were removed from the flasks with trypsin/EDTA, washed, and then lysed as described in Experimental Procedures. Fractions from about 1.5×10^6 cells were loaded onto each track. Samples were electrophoresed under non-reducing conditions on a 10% SDS−polyacrylamide gel and then electrophoretically transferred to nitrocellulose membrane. The membrane was then probed with a cocktail of G7, 78E, and 41D MAbs (which only recognize human clusterin). Bound MAb was detected with a sheep-anti-mouse Ig-HRP conjugate, followed by ECL. Lane 1, 320 ng of purified human serum clusterin; lane 2, total cell fraction, untreated controls; lane 3, total cell fraction, TNFα-treated; lane 4, cytoplasmic fraction, untreated controls; lane 5, cytoplasmic fraction, TNFα-treated; lane 6, nuclear fraction, untreated controls; lane 7, nuclear fraction, TNFα-treated. (B) Flow cytometry histograms showing the level of cell-associated clusterin for ethanol-permeabilized L929-pRc.clus cells that had been exposed to TNFα for 48 h. Dead cells (that in culture lacked an intact cell membrane) were discriminated on the basis of high red fluorescence (staining with 7-AAD; see Experimental Procedures). Cell-associated clusterin was detected as described in Experimental Procedures and is represented by green fluorescence (dead cells, thick line; cells that in culture had an intact cell membrane, thin line). The dotted line represents dead cells that were stained with DNP-9 (an isotype-matched control antibody) then SaMIg−FITC. Staining with DNP-9 of cells that in culture had an intact cell membrane was not significantly different to the dotted line shown. (C) Immunoblot of various cell fractions prepared from L929-pRc.clus cells that had been exposed to 100 ng/mL TNFα for 48 h, or untreated controls. Details are as for (A) except that all samples were electrophoresed under reducing conditions. The identity of the samples analyzed in each track are the same as in (A). The position of two clusterin bands at about 36 and 38.5 kDa in the total cell and cytoplasmic fractions prepared from TNFα-treated cells (lanes 3 and 5, respectively) are indicated with arrows.

The two expression plasmid systems used in our study (Lac Switch and pRc/CMV) do not allow "graded" expression of proteins. Thus, we were only able to test for the effects of two discrete levels of clusterin expression. Therefore, it may be that a level of clusterin expression considerably less than that produced in L929-pRc.clus cells (0.2 pg/cell/48 h) would be sufficient to provide protection against TNFα and potentiate the cytotoxicity of TGFβ; this has not been tested.

We have not excluded the possibility that clusterin overexpression may produce effects on the TNFα and TGFβ signaling pathways by virtue of some nonspecific chemical or physical property of clusterin protein. In this context it would be valuable to quantify the level of expression of endogenous clusterin protein during exposure to TNFα, against which to compare the levels of human clusterin expression occurring in the transfectants. However, as murine-specific anti-clusterin antibodies are currently not available, this has not been possible for L929 cells. In support of the contention that expression of clusterin can protect cells from TNFα-mediated cytotoxicity, Sensibar et al. have shown that anti-sense clusterin mRNA enhances sensitivity of LNCaP cells to TNFα, and that similar protection is provided by overexpression of clusterin in the same cell type (Sensibar et al., 1995). Interestingly, they showed that a transfected clone expressing low levels of clusterin was not protected from TNFα, while a clone that expressed higher levels was given complete protection. The observation that only higher levels of clusterin expression afford protection against TNFα agrees with our results in L929, where protection from TNFα is absent in the low-expressing induced L929-LS.clus cells but is present in the high-expressing L929-pRc.clus cells. Sensibar et al. did not report any effects of clusterin expression on cell death mediated by agents other than TNFα (Sensibar et al., 1995).

Our results demonstrate that overexpression of clusterin in L929-pRc.clus cells also results in some potentiation of the cytotoxicity of TGFβ (Figure 6). Although the suggestion from our results that high-level clusterin expression may potentiate the cytotoxicity of TGFβ on the one hand, yet on the other hand provide protection from TNFα-mediated cytotoxicity might at first appear paradoxical, the ability of a single type of molecule to be involved in signaling for the opposing outcomes of cell death versus survival is not unprecedented. Both Fas and TNFα induce cell death in tumor cells and lymphocytes but can also enhance cell proliferation in these same systems (Ware et al., 1996). Furthermore it is known that TNFα induces resistance to its own cytotoxic effect (Ware et al., 1996).

We have shown that in transfected L929-pRc.clus cells, overexpression of clusterin provided specific protection against death induced by TNFα or TNFα plus actinomycin

D, but not against death induced by several other agents tested (Figures 4A,B and 5). In addition, we have shown that protection against TNFα cytotoxicity produced by overexpression of clusterin in L929-pRc.clus cells appears to arise from an intracellular action of the protein. This protection is not produced by molecules secreted by L929-pRc.clus cells into the culture medium (Figure 7A) and cannot be mimicked by the addition of exogenous clusterin (Figure 7B). The protection cannot be ascribed to clusterin bound to the cell surface, since no surface clusterin was detected by flow cytometry, or to clusterin secreted by the cells and bound to the surface on which the cells were cultured (see above).

Using the two-hybrid system and other techniques, clusterin has recently been identified as binding with the intracellular domains of TGFβ receptors type I and II (Reddy et al., 1996b). It has also been independently demonstrated that in L929 cells TGFβ1 induces resistance to TNFα-mediated cytotoxicity (Chang, 1995). It was reported that the exposure of L929 cells to TGFβ1 induces phosphorylation of a variety of cellular proteins and the secretion of proteins that bind to the extracellular matrix. Both of these induced effects were implicated in protecting L929 cells from TNFα-mediated cytotoxicity (Chang, 1995). We have confirmed the work of others that exposure of L929 cells to TGFβ protects the cells from cytotoxicity associated with subsequent exposure to TNFα (Figure 6). In addition, we have shown that the high level of clusterin expression in L929-pRc.clus cells potentiated the cytotoxicity of TGFβ, and also that in the same cells pre-exposure to TGFβ produced no effect on the already relatively high resistance to TNFα-mediated cytotoxicity (Figure 6). These results indicate that the protective effects of clusterin overexpression and TGFβ against TNFα-mediated cell death are not additive and suggests that both clusterin and TGFβ may antagonise the TNFα-mediated cell death pathway via a common mechanism.

A truncated, non-glycosylated 43 kDa form of clusterin lacking the first 33 N-terminal amino acids and hence the hydrophobic signal peptide has been reported in TGFβ-treated HepG2 and CCL64 cells (Reddy et al., 1996a). This shorter form of clusterin is clearly translated on free ribosomes and is not translocated into the lumen of the endoplasmic reticulum (Reddy et al., 1996a). This protein could, therefore, interact with the cytoplasmic domains of TGFβ (or other) receptors. We have already excluded the possibility that secreted proteins play a role in the resistance of transfected L929 cells expressing clusterin to TNFα (see above). As a first step in examining the possibility that a cytosolic form of clusterin might interact with cytoplasmic domains of receptors to influence the cellular response to TNFα, we analyzed the forms of clusterin present in lysates of transfected L929 cells. We showed that, when analyzed under non-reducing conditions, cell lysates of L929-pRc.clus and IPTG-induced L929-LS.clus cells contain two major species of clusterin, one is similar to the secreted form with a molecular weight of about 80 kDa, and the other is an uncleaved form of apparent molecular weight about 64 kDa (Figure 8).

Similar forms of clusterin, at 58 and 70 kDa, were reported as present in lysates of HepG2 (Burkey et al., 1991). The 70 kDa form was shown to represent mature glycosylated clusterin, processed via the endoplasmic reticulum and Golgi systems, and internally cleaved to produce the two subunits. It has been suggested that this form is found within the cell inside Golgi and post-Golgi vesicle compartments. The 58 kDa form has been shown to represent a non-glycosylated, uncleaved precursor form to the 70 kDa species (Burkey et al., 1991). Two forms of intracellular clusterin have also been identified in MDCK cells: an uncleaved, incompletely glycosylated 65 kDa form and a cleaved and fully glycosylated 80 kDa form (Urban et al., 1987). The two forms of clusterin we have shown in L929 cell lysates almost certainly correspond to the intracellular forms described in HepG2 and MDCK cells (Burkey et al., 1991; Urban et al., 1987). In light of the recent demonstration of a novel 43 kDa truncated form of clusterin in TGFβ-treated HepG2 and CCL64 cells and the association of this form with the cell nucleus (Reddy et al., 1996a), we examined whether exposure of L929 cells to TGFβ or TNFα induced any changes in the level of synthesis of different molecular forms of clusterin or their association with the nucleus.

We showed that in response to TNFα, L929-pRc.clus cells demonstrate an increased association of fully processed clusterin, physically indistinguishable from cell-secreted clusterin, with the nucleus (Figure 9A). We used flow-cytometric analysis to demonstrate that there is an increased association of clusterin specifically with dead cells lacking an intact cell membrane (Figure 9B). This most probably results from secreted clusterin binding to dead cell nuclei. Our demonstration that BSA also binds to dead cell nuclei under these same conditions indicates that the interaction of clusterin with the nucleus is in this case unlikely to have physiological relevance. Our results also showed that an intracellular form of clusterin detected on immunoblots (following SDS/PAGE under reducing conditions) as a pair of bands at about 36 and 38.5 kDa was found in increased amounts in L929-pRc.clus cells treated with TNFα, TGFβ, azide, colchicine or staurosporine. Clusterin that is constitutively produced in either untreated or TNFα-treated L929-pRc.clus cells appears on the same immunoblots as a pair of poorly resolved bands at about 43−45 kDa (Figure 9C). The clusterin variant we report here is clearly not the truncated 43 kDa unprocessed form reported associated with nuclei in TGFβ-treated HepG2 and CCL64 cells (Reddy et al., 1996a). The TNFα-induced form of clusterin in L929-pRc.clus cells is internally proteolytically cleaved and is not specifically associated with the nucleus. An exon-skip mechanism has been hypothesized to account for a truncated clusterin mRNA in rat tissues treated with heat-shock or inhibitors of protein synthesis (Kimura & Yamamoto, 1996). Therefore, the apparently smaller size of the clusterin subunits induced by toxic agents could result from a variety of mechanisms, including modifications to transcription (Kimura & Yamamoto, 1996) or translation (Reddy et al., 1996a), additional proteolytic processing, or changes in glycosylation. Since the proportion of clusterin detected as the 36/38.5 kDa form was increased in response to all of the toxic agents tested, there is no specific indication that this novel form is involved in providing cells with protection from TNFα. However, it remains a possibility.

In summary, our results indicate that overexpression of clusterin can protect transfected L929 cells from death mediated by TNFα but not from death induced by colchicine, staurosporine or azide. Our results also indicate that high level expression of clusterin potentiates the induction of cell death in L929 cells by TGFβ. While we have not rigorously excluded the possibility of nonspecific effects of high levels

of clusterin protein, our results are compatible with the suggestion that clusterin and TGFβ may act through a common mechanism to protect L929 cells from TNFα cytotoxicity. We have shown that an intracellular action of clusterin protein is responsible for the protection against TNFα cytotoxicity but that this cannot be accounted for by synthesis of a 43 kDa truncated form of clusterin reported in other systems, or a cytokine-induced specific association of clusterin with the nucleus. We have recently shown that clusterin binds with high affinity to glutathione-*S*-transferase (S. B. Easterbrook-Smith, unpublished). It has been reported that the toxicity of TNFα in L929 cells involves the production of reactive oxygen species (ROS) (Schulze-Osthoff et al., 1994). Acting together, glutathione and GST comprise a ubiquitous cellular defense system against the effects of various types of metabolic stress, including ROS. It is known that GST can be induced by ROS; this may provide an adaptive response as GST can detoxify some of the harmful metabolites produced within the cell by oxidative stress [reviewed by Hayes and Pulford (1995)]. We speculate that clusterin may exert an intracellular effect on GST, increasing the ability of the glutathione/GST system to defend the cell against TNFα-induced ROS. In conclusion, it appears possible that under certain conditions clusterin may be able to selectively affect transduction pathways regulating cell death signals. Elucidation of the molecular processes underlying this putative intracellular function will require further investigation.

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