Clusterin Activates Survival through the Phosphatidylinositol 3-Kinase/Akt Pathway*

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Clusterin is, in its major form, a secreted heterodimeric disulfide-linked glycoprotein (75–80 kDa). It was first linked to cell death in the rat ventral prostate after androgen deprivation. Recent studies have demonstrated that overexpression of clusterin in prostatic cells protects them against tumor necrosis factor-α (TNFα)-induced apoptosis. However the details of this survival mechanism remain undefined. Here, we investigate how clusterin prevents cells from undergoing TNFα-induced apoptosis. We established a double-stable prostate cell line for inducible clusterin by using the Tet-On gene expression system. We demonstrated that 50% of the cells overexpressing clusterin escaped from TNFα- and actinomycin D-induced cell death. Moreover we demonstrated that the incubation of MLL cells with conditioned medium containing the secreted clusterin or the supplementation of purified clusterin in the extracellular medium decreased the TNFα-induced apoptosis significantly. This extracellular action implicates megalin, the putative membrane receptor for clusterin to mediate survival. Indeed clusterin overexpression up-regulated the expression of megalin and induced its phosphorylation in a dose-dependent manner. We interestingly showed that clusterin overexpression is associated with the up-regulation of the phosphorylation of Akt. Activated Akt induced the phosphorylation of Bad and caused a decrease of cytochrome c release. These results enable us to pinpoint one mechanism by which secreted clusterin favors survival in androgen-independent prostate cancer cells, implicating its receptor megalin and Akt survival pathway.

Clusterin, also known as testosterone-repressed message-2, is overexpressed in the rat prostate during castration-induced programmes cell death (1). Clusterin overexpression reaches a maximum 3–4 days post-castration and coincides with the onset of massive cell death (2). The clusterin level also rises in various tissues during cell death responses (3–5). As clusterin is present during apoptosis, it was initially viewed as a cell death inducer, but other studies suggest that clusterin overproduction occurs in resistant cells (6, 7). Thus, clusterin has been described as an anti-apoptotic factor, and it has also been implicated in prostate cancer progression to androgen independence (8). Miyake et al. (9) have demonstrated that the overexpression of clusterin in human androgen-responsive prostate cancer cells LNCaP by stable transfection rendered them highly resistant to androgen ablation, and the introduction of antisense testosterone-repressed message-2 oligodeoxynucleotide therapy in the Shionogi tumor model induces apoptosis and tumor regression. Moreover small interference RNA-mediated clusterin gene silencing in osteosarcoma and prostate cancer induces significant reduction of cellular growth and increases cellular apoptosis (10, 11).

Clusterin is a sulfated glycoprotein encoded by a single gene. It has two known isoforms obtained by alternative splicing (12). The first isoform, secretory clusterin (sClu) 3 is obtained by translation from the first AUG. The translation product of the clusterin gene is a polypeptide of 449 amino acids, where the first 22 amino acids represent the classical hydrophobic secretory signal sequence. Maturation of the primary translation molecule includes disulfide bonding, conversion to a high mannose endoplasmic reticulum-associated form of 60 kDa, extensive additional N-linked glycosylation, and finally, proteolytic cleavage in the trans-Golgi compartments that results in the mature secreted heterodimeric clusterin protein form of 70–80 kDa. The other clusterin isoform is the nuclear form (nClu) of 55-kDa, which originates after apoptosis induction from a 49-kDa primary product that is translated from an alternatively spliced Clu transcript. The elucidation of the function of clusterin after stress induction is clearly complicated by the discovery of the two isoforms of the protein. Theses isoforms explain in part the conflicting results for clusterin function in different laboratories. Many studies have demonstrated that overexpression of the nClu acts as a prodeath signal, inhibiting cell growth and survival (12, 13). However, other studies (14–16) have shown that the expression of the secreted form exerts cytotoxic properties. Despite growing evidence that the secreted clusterin acts as an anti-apoptotic factor and plays an important role in resistance to chemotherapeutic drugs (17), the molecular mechanism of clusterin-mediated survival signaling in cancer cells is not completely understood. Clusterin has long been viewed as a chaperone exerting its survival effect and cryopro-

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3 The abbreviations used are: sClu, secretory clusterin; nClu, nuclear clusterin; TGF, tumor growth factor; MLLTet-sClu, MatLyLu stable for the tetracycline transactivator factor and the secreted form of the clusterin; PI3K, phosphatidylinositol 3-kinase; NFκB, nuclear factor κB; EGFP, enhanced green fluorescent protein; Luc, luciferase; MT3, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BrdUrd, bromodeoxyuridine; FACS, fluorescence-activated cell sorter; IKK, inhibitor of κB kinase; IκB, inhibitor κB; IAP, inhibitor of apoptosis; Dox, doxycycline.

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tective function by at least two mechanisms: by increasing the solubility of denatured proteins and by mediating uptake and degradation of a broad spectrum of hydrophobic denatured aggregated molecules (18, 19).

Although clusterin has long been proposed to participate in cell survival and it has been extensively studied to inhibit TNFα apoptosis, no studies have been carried out to investigate the link between clusterin and survival signaling pathways. Here we have established and characterized a model cell line showing inducible production of the secreted form of clusterin, to study how this protein can protect cells against tumor necrosis factor-α (TNFα)-induced apoptosis. We have shown that when overexpressed or added in the culture medium, clusterin protects the MLLTet-sClu cell line from TNFα-induced apoptosis.

On the one hand we have studied how the overexpression of the secreted clusterin affects two major survival pathways that might be stimulated during TNFα-induced apoptosis. The phosphatidylinsositol 3-kinase (PI3K)/Akt (protein kinase B) pathway, one of the major survival pathways in the cell that is often constitutively activated in advanced stages of prostate cancer (20–22), and the NFκB survival pathway, known to enhance the proliferation and growth of many tumor cells, have been shown to increase activity in LNCaP cells to promote cell survival (23). On the other hand, we have studied the clusterin putative receptor, megalin (also called gp330), a 600-kDa membrane protein participating in debris-dependent clusterin recognition (24).

EXPERIMENTAL PROCEDURES

Materials—The oligonucleotides were synthesized by Invitrogen Life Technology (Grand Island, NY). Peptides used to generate antibodies were synthesized by NeoSystem (Strasbourg, France). Antibody against a synthetic C-terminal megalin peptide (CDETFKTNLVKEDSDV (25) and an N-terminal cysteine residue for conjugation to keyhole limpet hemocyanin) was prepared in rabbits at the Animal Facilities of the University of Liège. The antiphosphorylated megalin was generated in rabbits against a synthetic peptide (4569–4579 sequence of rat megalin plus the N-terminal cysteine residue for conjugation to keyhole limpet hemocyanin).

Cell Culture Conditions—The rat prostate cell line Dunning R3327 MAT LyLu was kindly provided by Dr. De Coster (Beerse, Belgium). Cells were maintained in RPMI supplemented with 10% Tet-System-approved fetal bovine serum (Tet-free FBS, Clontech, Palo Alto, CA), 50 units/ml penicillin (Invitrogen), and 50 μg/ml streptomycin (Invitrogen).

Expression Vectors and Generation of Transfected Cell Lines—The Tet-On System (Clontech), a modification of the tetracycline-controlled transcription activation system developed by Gossen and Bujard (26), was chosen to induce the conditional oversynthesis of clusterin by doxycycline (Dox). This system includes (i) the regulator plasmid pTet-On, expressing the gene coding for the reverse tetracycline-responsive transcriptional activator protein (under the control of the strong cytomegalovirus promoter) and the neomycin-resistance gene allowing selection of stably transformed cells in the presence of G418 (Geneticin); (ii) the response plasmid pTRE, containing the tetracycline-responsive element (TRE), into which the gene of interest was cloned. Because plasmid pTRE does not have a selectable marker, cells have to be cotransfected with pTK-HYG to permit selection of stable transformants by growth in the presence of hygromycine; (iii) two plasmids, pTRE-EGFP (containing a gene coding for an enhanced green fluorescence protein) and pTRE-Luc (a luciferase expression vector), provided an essential control in screening process of clonal cell lines. Plasmid pTRE-sClu contains, at its EcoRI site, the complete open reading frame of clusterin, excised from the pSB28 plasmid (kindly provided by Dr. S. Bettuzzi (1)) by means of the EcoRI.

For transfection the Lipofectamine PLUS™ reagent (Invitrogen) was used. 3 × 10⁶ MAT LyLu cells were transfected with 3 μg of pTet-On. After a 24-h culture, cells were trypsinized and seeded into 10-cm dishes (2 × 10⁵ cells/dish). 24 h later, cells were cultured in a medium containing 400 μg/ml G418 (Geneticin, Invitrogen) for 4 weeks. G418-resistant cells were isolated with a Freshney cylinder, and the activity of the transactivator system was estimated by transient transfection with pTRE-EGFP or pTRE-Luc. Clones showing the most intense green fluorescence in the presence of 2 μM Dox (a tetracycline derivative (Sigma-Aldrich)) were transiently transfected with pTRE-Luc to allow selection of the clone with the lowest basal expression level.

3 × 10⁶ cells of MLLTet were selected and cotransfected with 10 μg of pTRE-sClu or the empty vector pTRE (used as negative control) and 1 μg of pTK-HYG. Transformants were cultured with 800 μg/ml hygromycin (Invitrogen) for 15 days. Surviving clones were then tested for Dox-inducible clusterin synthesis by Western blot analysis as described below, using a monoclonal antibody against clusterin (Upstate, Charlottesville, VA).

Growth Rate Analysis—MAT Ly Lu (MLL) and MLLTet-sClu cells were seeded at 10⁵ cells per 35-mm diameter dish in RPMI containing 10% Tet-free FBS replaced 24 h later with medium containing 0.1% or 10% Tet-free FBS with or without Dox. The culture medium was replaced every 24 h. At daily intervals cells were detached with trypsin-EDTA (Calbiochem, San Diego, CA), stained with Trypan Blue (Sigma), and counted with a hemocytometer in triplicate.

MTT Cell Viability Assay—The MTT assay is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide), into a formazan precipitate, which is then dissolved in DMSO and quantified by spectrophotometry at 570 nm (27). MLL or MLLTet-sClu cells were seeded into 96-well dishes (3000 cells per well) in RPMI containing 10% Tet-free FBS and incubated for 48 h in the presence of varying concentrations of exogenous clusterin purified from rat serum as described below. Apoptosis was induced by adding 50 ng/ml TNFα (Roche Applied Science) and 4 μg/ml actinomycin D (Sigma) for 24 h. The cells were then tested for MTT conversion, and cell viability was estimated as the amount of MTT dye converted relative to that of control cells.

Cell Proliferation—Cell proliferation was quantified by measuring bromodeoxyuridine (BrdUrd) incorporation during DNA synthesis using the Cell Proliferation ELISA, BrdU kit (Roche Applied Science). The experiments were performed according to the manufacturer’s protocol.
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_Cells were seeded into 6-well plates. Clusterin production was induced with 2 μM Dox (control cells and cells where induction was not desired were cultured without Dox). Following treatment with 50 ng/ml TNFα and 4 μg/ml actinomycin D for 24 h, the number of apoptotic, necrotic, and viable cells was determined using an Annexin V-fluorescein isothiocyanate detection kit (MEBCYTO-Apoptosis kit, MBL, Immunosources, Halle-Zoersel, Belgium). Briefly, cells were trypsinized, washed with phosphate-buffered saline, and stained with annexin V and propidium iodide (PI) for 15 min at room temperature. Stained cells were quantified using a fluorescence-activated cell sorter (FACSCalibur, BD Biosciences) and CellQuest software (BD Biosciences). Apoptosis is often analyzed using fluorescent annexin conjugates to detect phosphatidylserine on the plasma membrane. The PI is a fluorescent vital dye that stains DNA. It does not cross the plasma membranes that are viable or in the early stages of apoptosis, because they maintain plasma membrane integrity and are permeable to PI. Annexin V binds to cells early in apoptosis and continues to be bound through cell death. PI is used to distinguish cells that are in the earlier stages of apoptosis (Annexin-positive and PI-negative) from those that are in later stages of apoptosis or already dead (necrosis, Annexin-positive and PI-positive). Each experiment was performed three times._

**RNA Isolation and Reverse Transcription-PCR**—Total RNA from induced and uninduced cells was isolated by a single-step guanidium thiocyanate method with Tri-Pure reagent. A 2-μg aliquot of RNA was reverse-transcribed for 45 min at 40 °C in the presence of 1 μg of random hexamers (Promega, Madison, WI) in a total volume of 20 μl. PCR mixtures contained 2 μl of reverse transcriptase (200 units/μl, Promega) in a total volume of 25 μl. Serial cDNA dilutions (1:1 to 1:100) were amplified for 20 cycles with sense (5′-ATTTCCCATATGATGACCT-3′) and antisense (5′-CGTAAAAAGATGACCCAGAT-3′) β-actin primers using an annealing temperature of 59 °C. Dilutions yielding equivalent amounts of product were amplified for 35 cycles with sense (5′-GGTTGGTGACGAGGAT-3′) and antisense (5′-AGTTGCAATTGCGCTCATCG-3′) megalin primers, and clusterin was amplified for 28 cycles with sense primers (5′-TGCTAGAAGTTCTACGCAG-3′) and antisense (5′-TTGGTGGCTGAAACGTCCAC-3′). The products of the PCR were separated by 1% agarose gel electrophoresis and visualized as described above.

**Immunoprecipitation**—Cells were washed once with phosphate-buffered saline. Then 1.5-ml ice-cold lysis buffer (25 mM HEPES, pH 7.7, 75 mM NaCl, 2.5 mM MgCl2·6H2O, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol, 20 mM α-glycerophosphate) containing 1× complete protease inhibitors (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, and the phosphatase inhibitors Na3VO4 (1 mM) and NaF (50 mM) was added, and the cells were then incubated at 4 °C for 10 min. Cells were disrupted by repeated aspirations through a 21-gauge needle and transferred to a 2-ml microcentrifuge tube. Cell debris was pelleted by centrifugation at 10,000 × g for 10 min at 4 °C. Supernatants were preclarified by adding 1 μg of normal rabbit IgG and protein G/A-agarose (Amersham Biosciences). The resulting supernatants were incubated with antibody for 2 h at 4 °C. Then 25 μl of protein G/A-agarose were added to the mixture, and the mixture was incubated overnight at 4 °C. Immunoprecipitates were collected by centrifugation and washed four times with lysis buffer. Finally the pellets were resuspended in 40 μl of 1× electrophoresis sample buffer and analyzed by Western blotting.

**Immunoprecipitation and Activity Assay of IkB Kinase (IKK)**—For the IKK assay, 106 MLL Tet-sClu cells cultured in T-75 flasks with or without 2 μM Dox for 48 h were treated for 10 min either with 50 ng/ml TNFα (Roche Applied Science), 4 μg/ml actinomycin D (Sigma), or both (each flask was treated separately). Then cells were collected with 400 μl of lysis buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The kinase from protein extracts (500 μg of protein/sample) was immunoprecipitated with 2 μg of anti-IKK α monoclonal antibody (Upstate) or the goat polyclonal anti-clusterin (SC-6419, Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with an anti-mouse or the anti-goat IgG coupled to hors eradish peroxidase. 4-Chloro-1-naphtol was used as a substrate according to the supplier’s instructions or alternatively the ECL (enhanced chemiluminescence) kit (Sigma-Aldrich) was used for the detection.

**For cytochrome c Western blot analysis, 50 μg of cytosolic protein extract, obtained as described by Yang et al. (28), was resolved by 14% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with a monoclonal anti-cytochrome c antibody (BD Pharmingen, San Jose, CA), and immunoreactive bands were visualized as described above.**
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clonal antibody (Sanvertech, Santa Cruz Biotechnology), and the resulting complex was incubated with protein A-agarose beads overnight at 4 °C (the beads were prewashed four times with lysis buffer). The in vitro kinase assay was performed by incubating the mixture at 30 °C for 30 min in a reaction buffer containing 1 µg of GST-1kBα-(1–55) (as substrate) and 100 µM ATP in ice-cold kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM α-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). The kinase reaction was stopped by adding 2× SDS sample buffer, and the products were electrophoresed on a 10% SDS-PAGE gel and transferred on a cellulose nitrate membrane. The phosphorylated 1kBα was detected by, using a monoclonal anti-phosphoserine 32–36-1kB (Westburg, Allison Park, PA) followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG. The immunoreactive band was then revealed by ECL reagents (Amersham Biosciences).

Electrophoretic Mobility Shift Assay—Poly nucleotide kinase (Promega) and [32P]ATP were used to label a DNA fragment corresponding to the NF-κB consensus sequence. The nuclear extracts obtained as described by Caccamo et al. (29) (10 µg) were incubated for 10 min in gel-shift binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 0.25 µg/µl poly(dIdC), 20% glycerol) and for 20 min at room temperature with the labeled fragment was added (radioactivity exceeding 50,000 cpm/ml). The samples were then resolved on a 6% polyacrylamide gel in 0.5 × Tris borate/EDTA buffer at 200 V. The gels were dried and developed by autoradiography.

RESULTS

Establishment and Characterization of a MATLyLu Cell Line Displaying Doxycycline-inducible Clusterin Synthesis—We first developed three double-stable MLL cell line clones displaying inducible clusterin synthesis. To this end, we used the Tet-On System kit (Clontech), a modification of the system developed by Gossen and Bujard (26), in which a gene coding for a tetracycline-regulated transcription activator and the gene to be expressed a negative control. Sixty hygromycin-resistant clones were transfected with pTRE-sClu or with the empty vector to establish a Tet-On MLL cell line. The screening step led to establishing a Tet-On MLLTet cell line. The screening results are shown in Fig. 2 displayed Dox dose-dependent clusterin production. When cells were treated with 2 µM Dox, production increased with the incubation time; the negative control clone (cells transfected with the empty vector) incubated for the same length of time in the presence of Dox did not show any accumulation of clusterin in the media (Fig. 2B).

Effect of Clusterin Overexpression on MAT LyLu Cell Growth and Proliferation—We next compared the growth of induced and uninduced MLLTet-sClu cells cultured in the presence of Dox (doses tested: 0.2, 1, 2, and 4 µM) for 72 h. Overproduction of clusterin had no cytotoxic effects. It did not affect cell viability, and it did not induce apoptosis (data not shown).

Cell counting and BrdUrd incorporation measurements did show somewhat reduced cell proliferation upon treatment with 2 µM Dox, as compared with cells maintained without Dox for 48 h. This effect was not due to the overexpression of clusterin, because the parental epithelial cells maintained in the presence extracts of MLLTet-sClu showed no immunoreactive band upon treatment with both upstate anti-clusterin antibodies, whether the cells were induced with 2 µM Dox or not. Even with the Santa Cruz Biotechnology antibody we have detected only a slight intracellular form when we have used the sensitive system of detection ECL after 10 min of exposition. These results show that the intracellular form is present but at a very low concentration compared to the secreted form, so we can deduce that our inducible cell line is a model where the major induced clusterin is secreted (Fig. 1). We can conclude that MLLTet-sClu cells represent a suitable model for studying the effects of the long form of this sulfated glycoprotein. The clone (Clone1) that expressed clusterin at a high level for which results are shown in Fig. 2 displayed Dox dose-dependent clusterin production. When cells were treated with 2 µM Dox, production increased with the incubation time; the negative control clone (cells transfected with the empty vector) incubated for the same length of time in the presence of Dox did not show any accumulation of clusterin in the media (Fig. 2B).
or absence of Dox showed similar proliferation (Fig. 3). We compared the proliferation rate of three clones overexpressing clusterin at a different level compared with the negative control and did not see significant effect on cell proliferation depending on the amount of clusterin synthesis. Also in Fig. 3 the proliferation rate of MLLTet-sClu clone 1 at different concentrations of Dox did not show any clusterin related effect.

**Secreted Clusterin Protects MLLTet-sClu Cells against TNFα-induced Apoptosis in a Dose-dependent Manner**—In the absence of Dox, treatment of MLLTet-sClu cells with 50 ng/ml TNFα and 4 μg/ml actinomycin D resulted in a significant percentage of cell death. After 24 h, ~60% of the treated cells, on average, were in early apoptosis (low PI staining) or late apoptosis (high PI staining). This cytotoxic effect was greatly alleviated when the MLLTet-sClu cells were pretreated for 24 h with 2 μM Dox: in this case, only 10% of the cells were found to die by apoptosis. The observed differences in resistance to TNFα treatment may thus reflect the ability of clusterin to delay the onset of apoptosis (Fig. 4A). We have also demonstrated that the clones displaying clusterin at different levels display differential sensitivity to the apoptotic effect of TNFα and actinomycin D, whereas the clone transfected with the empty vector did not show any protection effect. We can easily note that the difference in percentage of protection between the clones is in correlation with the quantity of clusterin produced in the media (Fig. 4C). We also studied the dose dependence of the observed cytoprotection. MLLTet-sClu cells were incubated without or with Dox (tested concentrations: 0.2, 0.5, 1, and 2 μM) for 24 h. The cells were treated with 50 ng/ml TNFα and 4 μg/ml actinomycin D for 24 h, collected, and analyzed by FACs after staining with Annexin V and propidium iodide. Fig. 4B shows that the higher the dose of Dox, the lower the percentage of apoptosis. Because Dox exerts no cytotoxic effect per se and because the amount of clusterin produced is Dox dose-dependent, it appears that secreted clusterin protects cells against TNFα-induced apoptosis in a dose-dependent manner.

**Effect of Exogenous Clusterin on TNFα-induced Apoptosis in the Parental Cell Line MATLyLu**—We then examined how TNFα and actinomycin D affect the parental cell line MATLyLu. As shown in Fig. 4D, the viability of MLL cells was found to decrease in response to treatment. Moreover, when purified rat clusterin was supplied exogenously to these cells, the effect diminished in a dose-dependent manner. At high clusterin concentrations the cytotoxic effect of TNFα was completely abolished (Fig. 4D). Moreover the incubation of the negative control cells transfected with the empty vector in medium containing sClu from induced MLLTet-sClu with 2 μM Dox for 48 h allowed the cells to become resistant to TNFα-induced apoptosis (Fig. 4E). Thus, the secreted form of clusterin acts from the outside of cells to enhance survival.
Megalin Binds to Clusterin and Its Expression Level Increases in Clusterin-overexpressing Cells—Megalin is a 660-kDa membrane protein described as an endocytic receptor of clusterin (24). By immunoprecipitation with anti-megalin followed by Western blotting with a monoclonal antibody against the clusterin/H9251 chain, we detected a protein having the molecular weight of the non-glycosylated clusterin alpha subunit (Fig. 5B).

Because the same antibody revealed no immunoreactive band in total protein extracts of Dox-induced MLLTet-sClu, it’s likely that the clusterin α chain is part of a megalin-clusterin complex. Because clusterin acts from outside the cell, we studied the expression of the megalin gene in clusterin-overproducing MLLTet-sClu cells. We first performed reverse transcription-PCR to measure levels of megalin mRNA. As shown in Fig. 5A, the level of megalin mRNA was higher in Dox-induced cells than in uninduced cells, and it rose in a dose-dependent manner. Next, megalin itself was detected by Western blotting in membrane-protein extracts of induced MLLTet-sClu cells, using a monospecific polyclonal antibody against the C-terminal peptide of the megalin, developed in our laboratory. Fig. 5C shows the immunoreactive band corresponding to megalin (molecular mass exceeding 250 kDa) in extracts of MLLTet-sClu cells overexpressing clusterin.

Clusterin also stimulated megalin phosphorylation as demonstrated by Western blotting using the antiphosphorylated peptide 4564–4579 sequence of rat megalin (Fig. 5D). This phosphorylation as shown in Fig. 5D decreased with the wortmannin treatment and was completely inhibited by 25 ng/ml after 8-h treatment, demonstrating that megalin phosphorylation is PI3K-dependent.

Clusterin Potentiates the PI3K/Akt Survival Pathway in MLLTet-sClu Cells—Given the importance of the PI3K/Akt survival pathway, it is important to examine how overproduction of clusterin affects this pathway in induced MLLTet-sClu cells. We therefore focused on three events: phosphorylation of Akt, phosphorylation of the Akt target protein Bad, and Bad-mediated cytochrome c release from mitochondria.

First, cells were cultured in the presence or the absence of Dox (doses tested: 0.2, 1, and 2 μM) for 48 h, and the amount of phosphorylated Akt was determined by Western blotting with a phospho-Akt-specific antibody, after prior immunoprecipitation of total Akt. Interestingly, the level of Akt phosphorylation was found to increase with the dose of Dox used to induce clusterin synthesis (Fig. 6).
We then evaluated phosphorylation of the Akt target protein Bad to check the correlation between Akt phosphorylation and Akt activity. The level of phosphorylated Bad was found to increase in parallel with Akt phosphorylation. Thus, Bad was not available to allow pore formation on the mitochondrial membrane. This is confirmed in Fig. 6A, which shows that the amount of cytochrome c released into the cytosol of MLLTet-sClu cells treated with TNFα/ActD for 12 h decreased as the concentration of Dox used to induce clusterin synthesis increased. These effects were caused by the overexpression of clusterin and not Dox itself, because the incubation of MLL cells with increasing concentrations of Dox had no effect on Akt phosphorylation (Fig. 6B).

**Effects of Wortmannin on Clusterin-mediated Cytoprotection against TNFα-induced Apoptosis**—The role of the PI3K as a major mediator of survival in prostate cancer cells is well documented. Exposure of MLLTet-sClu cells to wortmannin (Sigma), a specific inhibitor of PI3K, resulted in rapid and complete loss of Akt phosphorylation (data not shown). When uninduced MLLTet-sClu cells were pretreated with 25 ng/ml wortmannin for 8 h and then apoptosis was triggered with TNFα plus actinomycin D for 24 h, all of the MLLTet-sClu cells died (Fig. 7A). In contrast, at least 40% of the Dox-induced cells survived under the same conditions. Thus, clusterin protects cells from apoptosis even when the PI3K is inhibited. Wortmannin does not interfere with the clusterin production as shown in Fig. 7A. Here, we used immunoprecipitation to study the interaction between clusterin and megalin after 4-h treatment with 25 ng/ml wortmannin. As shown in Fig. 7B, after immunoprecipitation with anti-clusterin (mouse antibody) and detection with anti-megalin (goat antibody) the interaction of megalin with clusterin was increased weakly in induced cells after treatment with wortmannin and TNFα compared with control cells without wortmannin. However the amount of phospho-megalin (detected with the anti-phospho-megalin rabbit antibody) increased in the complex with clusterin in induced cell extracts without wortmannin treatment compared with the cells induced and treated with TNFα and wortmannin. When we immunoprecipitated the extracts with anti-megalin (a goat antibody) and then detected the clusterin with the polyclonal antibody (rabbit anti-clusterin) we have shown by Western blotting and immunodetection with the anti-rabbit-horse-radish peroxidase conjugate that the amount of clusterin increased in both inducible cell extracts with or without wort-
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Our results confirm that the PI3K/Akt survival pathway is important in the androgen-independent MLL rat prostatic cell line, because all cells die when the PI3K is inhibited. They also show that, although the survival effect of clusterin does appear to be linked to its effect on this pathway, there may be additional pathways PI3K independent through which clusterin enables MLLTet-sClu cells to resist TNFα-induced apoptosis.

Clusterin Does Not Affect NFκB Activity in MLLTet-sClu Cells—NFκB, like Akt, interferes with apoptotic pathways. Recent evidence suggests that the anti-apoptotic activity of Akt may be mediated by IKK-catalyzed phosphorylation. IKK phosphorylates IκB, causing its degradation and the release of NFκB. This factor is then translocated into the nucleus where it promotes cell survival by activating transcription of survival factors like survivin and IAP (30). We therefore investigated the possibility that NFκB might play a role in protection against TNFα-induced apoptosis.

First we studied the effect of clusterin overproduction on TNFα-induced apoptosis. FIG. 8 shows the results of in vitro kinase assays performed as described under “Experimental Procedures” on preparations obtained from MLLTet-sClu cells induced or not to overproduce clusterin and treated with TNFα, actinomycin D, or both. Induction of clusterin overexpression did not affect IKK activity. We also examined by Western blotting the amount of IκB present in cells under these conditions and in gel-shift assay the amount of active NFκB. The results of these experiments, also shown in FIG. 8, suggest that in our cell model, overexpression of the secreted form of clusterin does not interfere with IκB stability or NFκB activity.

Statistical Analysis—All presented assays have been repeated for at least three times, unless otherwise indicated. Each data point represents the mean of the independent experiments, and bars denote ± S.D. To determine the level of significance statistical evaluation was calculated by using the Student t test; p < 0.05 was considered to be significant.

DISCUSSION

Taken together our data confirm the survival effect of the secreted form of clusterin and point to a new mechanism by which sClu protects prostate cells from TNF-induced apoptosis. This mechanism implicates megalin and the Akt signaling pathway. To elaborate these conclusions we studied the survival effect of clusterin in an inducible prostate cell line. For that, we have established double-stable cell line clones displaying Dox-inducible synthesis of clusterin. Our choice for the inducible Tet-On gene expression system was to avoid the effects due to the clonogenicity of stable transfectants as described by Scaltriti et al. (31). Here, we have shown that MLLTet-sClu clones did not exhibit any significant morphological differences compared with the control cells. They did not show any alteration of the
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proliferation rate with the overexpression of clusterin. In contrast Scarliti et al. have shown that the overexpression of the intracellular form of clusterin induces G2/M phase arrest and cell death in PC3 prostate cancer cells. This reinforces our model where the secreted form is dominant and the intracellular form is only slightly expressed. Moreover, we have shown that the overexpression of sClu protects cells from TNFα and actinomycin D induced apoptosis, and this protection depends on the Dox dose used for the induction. To confirm that the survival effect induced by the overexpression of clusterin observed in this model is not due to the clonogenicity of one stable clone, we have compared three clones displaying Dox-induced clusterin. We have shown that the protection effect observed is dependent on the clusterin expression level. We have also compared these positives clones to a stable clone with the empty vector. These effects were completely absent in the control clone.

Although the protective effect of clusterin in prostatic cells has been demonstrated and studied extensively by several authors (17, 32, 33), the mechanism by which clusterin acts on cell survival is poorly understood. Here we have shown that clusterin acts as an extracellular survival signal, because the addition of the latter to the culture medium protects cells from TNF-induced apoptosis. These results are in agreement with those of Sintich et al., showing that the sensitivity of LNCaP cells to TNFα decreased in the presence of exogenous clusterin and that addition of an anti-clusterin antibody to PC3 cells sensitized them to TNF-dependent apoptosis (34). The findings of Trougakos et al. consolidate our observation. Indeed they show that the incubation of cells in media with a high concentration of sClu can protect them from the cytotoxicity of the intracellular clusterin and the neutralization of sClu by using specific antibodies sensitized cells to genotoxic stress (35). However, these results are in contrast with those of Zhang et al. (36) who have demonstrated that the survival effect of clusterin is due only to the intracellular form and the addition of exogenous recombinant clusterin to the culture media does not protect the human fibrosarcoma cell line from etoposide induced-apoptosis. These differences could be explained by the different systems used. Moreover clusterin function during cell death depends on the cellular context, the site of action, and the type of the signal that induces apoptosis.

The survival effect has been already attributed to the secreted form. An extensive number of studies have demonstrated that the tumor cell survival and the progression toward high grade and metastasis carcinoma were connected to the up-regulation of the secreted form of clusterin and the loss of the nuclear form (12, 15, 37). In prostate cancer the relationship between the sClu expression and the development of androgen resistance have been reported by different researchers (5, 9, 16, 17, 32, 38, 39). Recent work has shown that osteosarcoma cells became resistant to chemotherapeutic drugs by the up-regulation of clusterin and that antibody neutralization of the extracellular isoform sensitized the cells to drugs that induce cell death (40). Very recently Carnevali et al. (14) have shown that clusterin is involved in the defense of the lung against effects of cigarette smoke. They demonstrated that the coincubation of fibroblasts with conditioned medium with the sClu significantly decreased the cellular oxidation caused by the cigarette smoke extract. These findings consolidate our observations, because the incubation of parental cells in the culture media of MLLTet-sClu after 48 h of incubation with Dox to induce clusterin overexpression protected cells from TNFa-induced apoptosis.

Interestingly, we demonstrated that the survival effect of clusterin on prostatic cells was mediated in part by activation of the PI3K/Akt pathway, because Akt phosphorylation increased in a Dox dose-dependent manner. The fact that the effect was dose-dependent would indicate that the clusterin effect was physiologically significant and not just due to the use of an access of Dox. In our experiments, activation of the PI3K/Akt pathway led to a dose-dependent phosphorylation of Bad and to diminished cytochrome c release from the mitochondria. These effects were inhibited by wortmannin used at a dose 25 ng/ml, which is known to specifically inhibit PI3K. These results are also consistent with the survival function of Akt: phosphorylated Bad prevents pore formation in the mitochondrial membrane, blocks cytochrome c release, and thus inhibits TNFa-induced apoptosis (41).

With our inducible cell line we have established a direct relationship between increasing levels of clusterin and activation of the Akt survival pathway. This observation correlates well with the parallel increases in clusterin synthesis and PI3K/Akt activity observed in the progression of prostate cancer to androgen independence (20, 21, 42, 43). Clusterin, however, seems to act also via a PI3K-independent pathway. When uninduced, MLLTet-sClu cells exposed to the PI3K inhibitor wortmannin showed 100% mortality upon treatment with TNFa. In contrast, when the same cells were induced to overproduce clusterin, some 40% of them survived under these conditions. These cells must escape apoptosis by recruiting mechanisms that depend on other biological properties or another mode of action of clusterin.

Clusterin is also known to have chaperone protein activity. It helps to keep the extracellular environment free of proteins with abnormal conformation. Clusterin and abnormal proteins form complexes, which bind to specific receptors and become endocytosed (18, 44).

Clusterin can also interact directly or indirectly with factors involved in cell survival. Recent studies have shown that clusterin can alter the stability of IkB and modulate the activity of NFkB. This transcription factor has been linked to the progression and survival of many cancers, including prostate cancer (23). The NFkB pathway is an important one, activated during TNFa-induced apoptosis. In our experiments, the overproduction of clusterin in MLLTet-sClu cells had no effect on IkB stability, the IKK kinase, or NFkB activity. These results contrast with those obtained by Santilli on LAN5 and human neuroblastoma cells (45). The discrepancy between our results and those of Santilli is due to the fact that our observations concern the secreted form acting from the outside, whereas Santilli’s observations essentially concerned the action of intracellular clusterin lacking the protein export signal. Furthermore these data confirm that our model is indeed a model for the secreted form of clusterin.

Because clusterin acts as an external survival signal, we focused on megalin as its possible site of action on the mem-
brane. We have shown here that megalin is indeed the receptor of clusterin in our model. To our knowledge it is the first time that megalin expression on the mRNA and protein level has been demonstrated in prostate epithelial cells. Binding of clusterin to this membrane protein is suggested by our observation that megalin and clusterin co-immunoprecipitate in the presence of anti-megalin antibody. Megalin or gp330 has been shown previously to be a clusterin receptor that binds clusterin in rat epididymal epithelia and in renal epithelial cells, with subsequent internalization (46, 47). Interaction between clusterin or ApoL and megalin has also been observed in Alzheimer disease, where it leads to formation of amyloid bodies and to their internalization by neuronal cells. Overexpression of megalin mRNA was observed in the MLLTet-sClu cell line upon induction of clusterin expression with Dox. These results were confirmed by Western blotting with an anti-megalin antibody. Up-regulated synthesis of a receptor in response to an increased concentration of the ligand is a common regulatory mechanism of targeted cells. This observation strengthens our hypothesis that clusterin acts as an extracellular signal via the megalin receptor. Indeed recent studies show that megalin can interact with protein kinase B and protect renal proximal tubule cells from apoptosis induced by the high level of albumin (48).

Megalin has long been viewed as an endocytic receptor involved in the internalization of different ligands (44, 49). Recent studies indicate that in addition to providing an endocytosis signal, the cytoplasmic tail of this receptor is crucial to the interaction of cytoplasmic adaptors with scaffold proteins and thereby mediates signal transduction (50, 51).

As early as 1996, cloning and sequencing of human megalin cDNA and protein alignments enabled investigators to identify on megalin a potential phosphorylation consensus sequence 4569–4579 capable of interacting with SH2 domains on PI3K (50, 51). Here we have demonstrated that the sequence 4569–4579 of rat megalin is a PI3K phosphorylation site and the phosphorylation of tyrosine 4573 is induced by clusterin. In addition, in the absence or presence ofwortmannin-specific inhibitor of PI3K, by immunoprecipitation we have shown that clusterin interacts better with the phosphorylated form of megalin and that clusterin may stabilize the megalin-clusterin complex via its interaction. Finally clusterin also increases the PI3K dependent phosphorylation of Akt leading to the activation of the survival pathway via Bad phosphorylation. This suggests that clusterin may act as an external survival signal via a transduction mechanism involving its megalin receptor and PI3K/Akt pathway. The elucidation of the details of this new mechanism will require further investigations.

In conclusion our data show for the first time that the overexpression of the secreted isoform of clusterin protects the prostate androgen-independent epithelial cells from TNF-induced apoptosis through the activation of the Akt survival pathway and via its receptor the megalin.

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