Clusterin Regulates Drug-Resistance in Melanoma Cells

Christoph Hoeller,* Barbara Pratscher,* † Christiane Thallinger, * Dorian Winter, ‡ Dieter Fink, ††§ Boris Kovacic, † Veronika Sextl, † Volker Wacheck, † Martin E. Gleave, § Hubert Pehamberger,* and Burkhard Jansen* ††§

Department of Dermatology, Division of General Dermatology and Medical University Vienna, Vienna, Austria; † Department of Clinical Pharmacology, Section of Experimental Oncology/Molecular Pharmacology, Medical University Vienna, Vienna, Austria; ‡ Center for Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria; † Prostate Centre, University of British Columbia, Vancouver General Hospital, Vancouver, Canada; * Department of Pharmacology and Toxicology, Medical University Vienna, Vienna, Austria

Clusterin has recently been shown to act as an antiapoptotic protein that confers drug-resistance in models of epithelial tumors. The aim of our work was to provide an insight into a possible role of clusterin in the regulation of drug-resistance in melanoma. In tissue samples, clusterin expression was low in nevi, but high in primary melanoma and melanoma metastases. Clusterin was also strongly expressed in melanoma cell lines, but was barely detectable in cultured melanocytes. To elucidate a possible role of clusterin in drug-resistance of melanoma, clusterin expression was regulated by either plasmid-driven overexpression or by antisense-mediated downregulation. Clusterin overexpression was associated with an increase in drug-resistance, i.e., with an increased survival of melanoma cells in the presence of cytotoxic drugs. In contrast, downregulation of clusterin by 2′-O-(2-methoxy)ethyl (2′MOE)-modified antisense oligonucleotides (AS-ODN) directed against clusterin mRNA significantly reduced drug-resistance, i.e., decreased survival of melanoma cells in the presence of cytotoxic drugs. To evaluate the effects of clusterin-antisense treatment in vivo, we applied an SCID-mouse/human-melanoma xenotransplantation model. Pre-treatment of mice with the 2′MOE-modified clusterin AS-ODN was associated with a significantly improved tumor response to dacarbazine as compared with animals pretreated with a scrambled control oligonucleotide. Taken together, we show that clusterin is strongly expressed in melanoma. Downregulation of clusterin reduces drug-resistance, i.e., reduces melanoma cell survival in response to cytotoxic drugs in vitro and in vivo. Thus, reducing clusterin expression may provide a novel tool to overcome drug-resistance in melanoma.

Key words: antisense oligonucleotides/apoptosis/clusterin/drug-resistance/melanoma


Melanoma is a highly aggressive neoplasm that shows a poor response to currently applied treatment regimens. The standard cytotoxic therapy, dacarbazine, has a response rate between 15% and 20% in the more optimistic studies and seldomly leads to durable responses. A number of combination therapies have also remained unsatisfactory (Serrone et al, 2000; Tsao et al, 2004). Hence, drug-resistance, defined as decreased cell death in response to a cytotoxic therapy, is a main problem in the therapy of metastatic melanoma. Drug-resistance can be modulated by increased efflux of a drug, increased metabolism/detoxification or an increased resistance of tumor cells against the induction of apoptotic cell death by cytotoxic drugs. In melanoma the latter is a major mechanism. Melanoma has a very low intrinsic level of apoptosis and different proteins in the apoptotic pathways can be defective by mutation or loss of expression. Alternatively, apoptosis can be regulated by other pathways, e.g., the regulation of Bcl-2 by ras-activated pathways, to achieve a high antiapoptotic potential (Rockmann and Schadendorf, 2003; Soengas and Lowe, 2003).

Clusterin is a protein widely expressed in mammalian tissues, and is also known as testosterone repressed message-2 (TRPM-2), sulfated glycoprotein 2 (SGP-2), or apolipoprotein J (ApoJ). It was first described as a protein isolated from ram testes fluid with the ability to “cluster” red blood cells (Blaschuk et al, 1983). Clusterin is synthesized as a primary, 60 kDa, translation product of 449 amino acids, which is cleaved into an α and β subunit. These subunits are subsequently linked by disulfide bridges to form an extensively N-glycosylated, secreted, heterodimeric protein of 76–80 kDa. The final molecular weight depends on the degree of glycosilation, and the α and β subunits usually appear as a smear around 40 kDa in an SDS polyacrylamide gel. Besides the mRNA transcript that codes for the heterodimeric form of clusterin, a second transcript coding for a smaller (50–55 kDa) isoform localized in the nucleus can be generated by alternative splicing (Leskov et al, 2003). The nuclear isoform is not cleaved into an α and β subunit or extensively glycosylated. It was shown to be located in the cytoplasm of MCF-7 breast carcinoma cells as an inactive precursor that translocates into the nucleus upon irradiation (Yang et al, 2000). Clusterin was implicated in tissue

Abbreviations: AS-ODN, antisense oligonucleotide; 2′MOE, 2′-O-(2-methoxy)ethyl; SC-ODN, scrambled control oligonucleotide
remodeling, lipid transport, regulation of complement lysis, stabilization of stressed proteins, and apoptotic cell death. A clusterin knockout mouse was fertile and had no obvious phenotype. (Rosenberg and Silken, 1995; Jones and Jomary, 2002; Trougakos and Gonos, 2002) A number of studies marked clusterin as an antiapoptotic protein that protects cells from diverse apoptosis-inducing stimuli like androgen withdrawal, heat shock, oxidative stress, stimulation of the CD95/Fas pathway, or treatment with ethanol (Viard et al, 1998; Miyake et al, 2000b, 2001; Dumont et al, 2002). In experimental animal models, inflammatory myocardial damage in autoimmune disease was increased in the absence of clusterin and neuronal cell death after experimentally induced cerebral hypoxia was decreased by overexpression of clusterin (McLaughlin et al, 2000; Wehrli et al, 2001). In contrast, the 50 kDa nuclear isoform of clusterin led to growth inhibition and caspase-3-independent cell death in MCF7 cells (Yang et al, 2000). In histological samples taken from normal colon mucosa, colon adenoma, and invasive colon carcinoma, a switch from preferential expression of the nuclear to the cytoplasmic isoform of clusterin was observed (Pucci et al, 2004).

In line with an antiapoptotic role of the heterodimeric isoform of clusterin is the observation that high clusterin expression was detected in various human tumors and in vivo tumor models (Parczyk et al, 1994; Miyake et al, 2000b; Wellmann et al, 2000; Xiaodi et al, 2003). Clusterin expression also positively correlates with increasing malignancy and pathological grading of tumors in breast and prostate cancer (Steinberg et al, 1997; Redondo et al, 2000). Clusterin was, however, also demonstrated to exhibit growth-inhibiting effects in in vitro models of SV40 immortalized or epidermal growth factor (EGF)-stimulated prostate cells (Bettuzzi et al, 2002; Zhou et al, 2002). In c-Myc-transfected epithelial cells, clusterin blocked carcinogenesis in vivo (Thomas–Tikhonenko et al, 2004).

Based on its antiapoptotic properties, clusterin became an interesting target for antisense oligonucleotide (AS-ODN)-based therapeutic strategies. Downregulation of clusterin led to decreased drug-resistance, i.e., decreased survival of tumor cells in response to a cytotoxic therapy, in tumor cell lines derived from prostate, kidney, and lung cancer (Miyake et al, 2000a; Zellweger et al, 2000; July et al, 2004). Direct comparison of a phosphorothioate-modified ODN with an additionally 2’-O-(2-methoxy)ethyl (2’MOE)-modified ODN (OGX-011, Oncogenex Technologies, Vancouver, Canada) showed a higher activity and a longer half-life of 2’MOE-modified ODN (Zellweger et al, 2001). Recently, OGX-011 entered clinical phase I testing for prostate and other clusterin-positive cancers.

The aim of our study was to find out whether clusterin is expressed in melanoma and whether clusterin would modulate drug-resistance in melanoma cells in vitro and in vivo.

Results

Clusterin is overexpressed in human melanoma cells as compared with melanocytes. As shown in Fig 1, both the uncleaved (60 kDa) and the β-subunit (40 kDa) of the mature form of heterodimeric clusterin were detected in all melanoma cell lines investigated, whereas they were barely detectable in two different batches of normal human melanocytes. A band correlating to the ~50 kDa isoform, described to be able to locate to the nucleus, was neither detected using the routinely applied goat polyclonal antibody (Fig 1) nor with a monoclonal mouse antibody recently described to be able to locate to the nucleus, was neither detected using the routinely applied goat polyclonal antibody (Fig 1) nor with a monoclonal mouse antibody recently shown to detect this isoform of clusterin (data not shown). To assess the expression in clinical samples, immunohistochemical staining was performed in a series of nevi, primary melanomas, and melanoma metastases. Although a low expression of clusterin was present in all samples, only two of 11 nevi (18%) had a high expression of clusterin, defined as more than two-thirds of cells staining positive for clusterin. In contrast, four of ten primary melanomas (40%) and six of ten melanoma metastases (60%) exhibited a high expression level of clusterin, indicating that expression of clusterin increases in the course of melanoma disease progression (data not shown).

Overexpression of clusterin leads to increased drug-resistance in melanoma cells. Clusterin overexpression was achieved using plasmid-driven stable transfection. Mel-Juso cells were selected for this experiment since they displayed the lowest melanoma-associated expression of clusterin (Fig 1, lane Mel-Juso). Expression of clusterin in wild-type (Mel-Juso), vector control (Juso-Neo), and clusterin-overexpressing (Juso-Cl) cells was verified by western blotting (Fig 2A). In a cytotoxicity assay, clusterin overexpression significantly increased resistance to cytotoxic drugs after treatment with cisplatin or taxol. S-fluorouracil (SFU) was also tested but did not have an effect on Mel-Juso cells (data not shown). Clusterin overexpression increased the IC50 for cisplatin from 6 to 27 μM and the IC50 for taxol from 2 to 15 μM (Fig 2B and C). Hence, we conclude that clusterin overexpression protects the cells from drug-induced cytotoxicity.

Antisense oligonucleotides (AS-ODN) efficiently downregulate clusterin and enhance sensitivity toward cytostatic drugs. If overexpression of clusterin is associated...
Calculated IC50 are indicated in the upper right corner of each panel.

We were interested in knowing whether a combined treatment of AS-ODN directed against clusterin and chemotherapeutics might decrease drug-resistance in vitro. Three different chemotherapeutics were used: (i) cisplatin, a drug forming mainly G–G inter- and intrastrand linkages, (ii) taxol, an antimicrotubule agent, and (iii) the antimetabolite 5FU. The different time frames applied in these assays reflect the different mechanisms of the drugs used. In the case of cisplatin, an IC50 of 9 μM was observed after pretreatment with 500 nM clusterin AS-ODN compared with an IC50 of 32 and 39 μM, respectively, after treatment with 500 nM of SC-ODN or lipofectin alone (Fig 3B). A similar shift in dose–response curves was achieved using a combination of AS-ODN and taxol or 5FU (Fig 3C and D). Hence, we conclude that AS-ODN treatment against clusterin significantly reduces drug-resistance of melanoma cells, independent of the respective cytotoxic mechanism.

Downregulation of clusterin is associated with increased activation of drug-induced apoptosis. We reasoned that the downregulation of clusterin would sensitize melanoma cells to the pro-apoptotic effect of cytotoxic substances. An early marker for apoptosis is an increase in cells exhibiting phophatidylserine on the outer leaflet of the cell membrane that can be measured by annexin V binding. Subsequently, these cells become permeable for DNA-binding drugs like 7-aminoactinomycin D (7AAD) and show reactivity for both markers (van Engeland et al, 1998; Le-coeure et al, 2002). Since Annexin V detects early phases of apoptotic cell death, we decided to analyze the cells 12 h after addition of cisplatin. Downregulation of clusterin by the AS-ODN in combination with a subsequent treatment with 10 μM of cisplatin was associated with a significant increase in annexin V binding whereas no difference was seen in the respective controls (Fig 4A and B). Interestingly, some cells seemed to react with apoptosis to treatment with clusterin-directed antisense only. (Fig 4A, lane control, lower panel and Fig 4B).

Downregulation of clusterin enhances the response to dacarbazine in vivo in an SCID-mouse/human-melanoma xenotransplantation model. To test whether the clusterin 2′MOE-AS-ODN would also influence the drug-resistance of melanoma cells in vivo, 518A2 melanoma cells were injected subcutaneously into the flank of SCID mice. Antisense treatment was started after formation of an established tumor of at least 100 mm2 tumor volume and was performed every three days, i.e., following a loading phase of 4 d with daily injections. Clusterin expression was reduced by >70% in tumor samples, 11 d after start of treatment (23 ± 5%) as compared with mice treated with SC-ODN (100 ± 15%) or saline (97 ± 22%) (Fig 5A and B). Downregulation of clusterin in the antisense-treated group was observed until the end of the experiment, 3 days after the last injection. No significant difference in tumor volume between animals pre-treated with the clusterin AS-ODN, the SC-ODN, or animals injected with saline alone was observed (Fig 5C, inset). In contrast, AS-ODN pre-treated mice demonstrated a reduction in tumor size by more than 85% after a 4-d course of daily treatment with 80 mg per kg dacarbazine. The tumors in the control groups did not show...
any reduction in size at this time point and reacted to the chemotherapy with a delay of 3–6 d (Fig 5C, large panel).

Mouse weight at the end of the in vivo experiment was not significantly different, indicating that there was no apparent difference in toxicity between the treatment groups (data not shown).

**Discussion**

Conflicting evidence exists with respect to clusterin’s role in cell death as well as to its role in carcinogenesis and tumor progression. Clusterin was presented to protect cells from apoptotic stimuli in vitro, and increased cell death was seen under some conditions in adult animals of a clusterin knockout mouse strain (Viard et al, 1998; McLaughlin et al, 2000; Miyake et al, 2000b, 2001; Wehrli et al, 2001; Dumont et al, 2002). The absence of clusterin was, however, also reported to lead to a decrease in neuronal cell death after experimentally induced cerebral hypoxia in fetal mice (Han et al, 2001). Clusterin was demonstrated to protect tumor cells from prostate, kidney, and lung cancer against apoptosis (Miyake et al, 2000a; Zellweger et al, 2000; July et al, 2004) but is also able to inhibit cell growth in tumor cells stimulated either by addition of EGF, or cells transformed by SV40 or c-myc (Bettuzzi et al, 2002; Zhou et al, 2002; Thomas-Tikhonenko et al, 2004). Although clusterin is upregulated in renal clear cell carcinoma, in some lymphomas, in prostate cancer, in breast cancer, and was identified as a marker in intestinal neoplasias (Parczyk et al, 1994; Miyake et al, 2000b; Redondo et al, 2000; Wellmann et al, 2000; Xiaodi et al, 2003), it is downregulated in testicular germ tumors (Behrens et al, 2001). This indicates that clusterin exhibits different functions depending on the cellular background and the specific situation, i.e., tumor initiation versus tumor progression or fetal mice versus adult animals.

This study was performed to investigate clusterin’s role in melanoma.

We demonstrate that clusterin is expressed at high levels in human melanoma. Levels of heterodimeric clusterin in melanoma cell lines are significantly upregulated as...
compared with melanocytes and increased expression is found in a higher percentage of primary melanomas and melanoma metastases than in melanocytic nevi. Comparable with our data, increased expression according to disease progression, was observed in breast and prostate cancer (Steinberg et al., 1997; Redondo et al., 2000).

We further demonstrate that clusterin modulates drug resistance in melanoma cell lines.

We show that overexpression of clusterin leads to an increase in resistance to cisplatin and taxol in melanoma cells with a low endogenous level of clusterin. Downregulation of clusterin by AS-ODN reduced the IC50 for cisplatin, taxol, and 5FU. This demonstrates that the effect achieved by the regulation of clusterin must be due to an influence on a common pathway activated by all of these different cytotoxic substances. An increase in apoptotic cell death as shown by an increase of cells staining positive for annexin V was observed after combined treatment with AS-ODN and dacarbazine.

**Figure 4**

Down regulation of clusterin increases drug induced apoptosis.

(A) 518A2 melanoma cells were pretreated with either lipofectin alone (LF), lipofectin in combination with 500 nM of an antisense oligonucleotide directed against clusterin (AS) or lipofectin in combination with 500 nM of scrambled control oligonucleotide (SC). After treatment, cells were stained with FITC-labeled annexin V and 7-aminoactinomycin D (7AAD). Subsequently, cells were analyzed by fluorescence-activated cell sorter (FACS). Numbers of annexin V positive (lower right) or annexin V/7AAD double positive (upper right) cells are given as % of events gated at the side of the charts. The experiment represents one of three that gave comparable results. (B) Results from the experimental protocol described in Fig 5A. Bars represent the mean number of annexin V positive cells from three independent experiments in % ± SD. Groups were compared by a paired t test and the respective p-values are indicated.

**Figure 5**

SCID-mouse human-melanoma xenotransplantation model. Mice with established tumors grown after s.c. injection of 518A2 melanoma cells were pre-treated with either 10 mg per kg clusterin antisense oligonucleotide, scrambled control oligonucleotide, or saline. Eleven days after start of treatment animals received 80 mg per kg of dacarbazine over 4 d. (A) Western blot for clusterin tumor in lysates at days 23 and 33. (B) Clusterin levels from three tumors per treatment group at day 11. Bands were analyzed densitometrically, normalized to the β-actin control, and depicted as % of SC-ODN. A mouse monoclonal antibody against clusterin was used for the immunoblot. Bars represent SD. (C) Mean tumor volume throughout the experiment. Control groups without dacarbazine treatment are shown in the insert. Bars represent SD. * * * represents differences statistically significant by Student’s t test (p < 0.05).
cisplatin. Hence, we conclude that clusterin regulates drug-resistance of melanoma cells by influencing drug-induced apoptosis. A smaller increase in annexin V staining was also observed after antisense-treatment alone in vitro, whereas no significant difference in tumor size between antisense-treated animals and animals treated with the SC-ODN without the addition of dacarbazine was seen.

The mechanism underlying clusterin’s regulation of apoptosis is not yet clarified. The expression of an isoform of clusterin that is able to translocate to the nucleus and upon doing so exerts pro-apoptotic functions, is, as mentioned in the introduction, one possibility (Yang et al., 2000; Leskov et al., 2003). We did not detect this 50 kDa isoform in melanocytes or melanoma cells. Although a switch from expression of the nuclear isoform to expression of the heterodimeric isoform of clusterin was observed to be associated with the progression from colon adenoma to colon carcinoma (Pucci et al., 2004), we did not detect such a difference between melanocytic nevi and melanoma samples. In an osteosarcoma cell line, downregulation of Bcl-2 was found to be associated with RNAi-mediated downregulation of clusterin (Trougakos et al., 2004). We did not observe Bcl-2 regulation after antisense-mediated downregulation of clusterin in melanoma cells (data not shown).

AS-ODN, chemically modified stretches of single-stranded DNA, are pharmacologically potent inhibitors of disease-related protein expression (Jansen and Zangemeister-Wittke, 2002). They are designed to bind to their complementary mRNA sequence once they get inside the cell, thereby inhibiting expression of the encoded protein (Myers and Dean, 2000). 2'MOE-modified AS-ODN have the advantage of increased stability and a longer half-life as compared with earlier generations of antisense molecules. Therefore they can be applied up to only once a week and are still as effective as conventional AS-ODN with a phosphorothioate backbone (Zellweger et al., 2001). We applied clusterin AS-ODN every 3 d, i.e., in an SCID-mouse/human-melanoma xenotransplantation model and observed a downregulation of clusterin in the tumor. This was associated with a significantly improved tumor response to dacarbazine as shown by a reduction of tumor size by more than 80% only 4 d after the initiation of cytostatic treatment. Control treated animals did not show any reaction at this time point and did only react with a delay of about 6 d.

Our data identify clusterin as a protein capable of regulating apoptosis and drug-resistance in model systems of human melanoma in vitro and in vivo. Together with data on the expression of clusterin in clinical samples of metastatic melanoma, this provides a basis for the further development of strategies downregulating clusterin to a clinically applicable therapeutic modality for patients suffering from melanoma.

**Materials and Methods**

**Tumor cell lines** 518A2, 607B (courtesy of Dr Peter Schrier, Leiden, the Netherlands), SKMEL28 (ATCC), and Mel-Juso (German Collection of Microorganisms and Cell Culture, DSMZ, Braunschweig, Germany) melanoma cells were maintained in DMEM (Invitrogen, Carlsbad, California) supplemented with 8% heat-inactivated FCS. 500 μg per mL Geneticin (Invitrogen) was used for selection of transfectants. Normal human epidermal melanocytes (NHEM) were obtained as cryopreserved cells from Clonetics/Cambrex (East Rutherford, New Jersey). NHEM were maintained in MM-2 medium supplemented with the MGMT-3 bullet kit (all Clonetics) and were used between the third and sixth doubling after thawing. For experiments comparing melanocytes and melanoma cells, all cells were cultured in DMEM with 8% FCS for 24 h.

**Generation of transfected Mel-Juso cells** The human clusterin cDNA containing mammalian expression vector pRc/CMV was kindly provided by Dr Martin Gleave (Vancouver, Canada). Cells were transfected using a cationic lipid (Lipofectin, Invitrogen) according to the manufacturer’s instruction. 500 μg per mL Geneticin (Invitrogen) was used for clonal selection of transfected cells. Individual clones were tested for clusterin expression by western blotting. To avoid subcloning artifacts, clones were pooled and used as a “mass culture” in the cytotoxicity assays.

**Clusterin oligodeoxyribonucleotides** 2′MOE-modified ODN’s used in this study were provided free of charge by OncoGenex Technologies (Vancouver, Canada). The sequence of the AS-ODN is CAGCAGCAGAGTCTTCATCAT (OGX-011); the sequence of the SC-ODN is CAGCGGTGACAACAGTTTCAT.

**Treatment of cells with ODN ± chemotherapy** Lipofectin (Invitrogen) was used to increase the ODN uptake of cells. Cells were treated for 4 h on two consecutive days with ODN in the indicated concentration in serum-free OPTIMEM (Invitrogen) containing 4 μg per mL Lipofectin. After this time, the medium was replaced with standard culture medium as described above. Chemotherapeutic agents included cisplatin, taxol, and 5FU and were added 24 h after pretreatment with the respective oligonucleotides. Cisplatin, taxol, and 5FU were obtained from the pharmacy at the university hospital.

**Western blotting** Samples were prepared using a buffer containing 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris, 2 mM PMSF, 2 mM Na3O4V, and complete protease inhibitors (Roche Diagnostics, Basel, Switzerland). Western blots were performed using 30 μg of protein. Prior to application, protein lysates were washed at 95 °C for 10 min in equal volumes of a 2× loading buffer (30 mM Tris, 12.5% glycerol, 1% SDS, 12.2 mM mercaptoethanol, 0.05% bromophenol blue). Ponceau-red stain and an antibody directed against β-actin or vinculin were used as loading controls. Antibodies used were: anti-clusterin goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, California), anti-clusterin mouse monoclonal (Lot 41D, Upstate, Charlottesville, Virginia) (used to control for expression of the 50 kDa nuclear isoform of clusterin), and anti-actin rabbit polyclonal (Sigma, St Louis, Missouri).

**Immunohistochemistry** 4 μm thick, paraffin-embedded tissue sections were deparaffinized using xylol and an ethanol dilution series. Melanin was bleached by incubation in 1% sodium nitrate and 2% sodium bisulphite. Antigen retrieval was performed by cooking in citrate buffer and endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide. After incubation in blocking serum, slides were incubated with an anti-clusterin rabbit polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:100. A rabbit isotype antibody was used as negative control. Staining was performed using the LSAB kit (DAKO, Glostrup, Denmark). The experiments using human material were approved by the local ethics committee.

**Cytotoxicity assays** The influence of AS-ODN treatment or overexpression of clusterin on chemosensitivity was assessed using an MTS-assay system (Cell Titer 96, Promega, Madison, Wisconsin). This assay measures the activity of dehydrogenase enzymes, which is roughly equivalent to the number of viable cells. Briefly, 2 x 10^5 cells per well were seeded in 96-well culture plates and allowed to adhere overnight. ODN treatment experiments were
performed as described above. Cells were then treated with various concentrations of cisplatin, taxol, or 5FU. After the indicated times, cell titer 96 reagent was added and cells were incubated at 36°C for 2 h. The absorbance was determined using a microtiter plate reader (Wallac/Perkin Elmer, Wellesley, Massachusetts). Absorbance values were normalized for the respective control and expressed as the relative amount of enzyme activity. The controls used were cells from the same pre-treatment group without addition of the respective cytotoxic substance measured at the same time point.

Fluorescence-activated cell sorter (FACS) analysis

Cells were seeded in 60 mm culture wells and treated with ODN as described above. Subsequently, cells were trypsinized, washed twice in PBS, and resuspended in a buffer containing 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4. Cells were then incubated with FITC-labeled annexin V (Bender Medsystems, Vienna, Austria) for 5 min and subsequently coincubated with 1 μg per mL 7AAD (Sigma) for another 10 min. Labeled cells were analyzed on a FACSScan (BD Biosciences, San Jose, California) with an argon laser at 488 nm.

SCID-mouse/human-melanoma xenotransplantation model

10^5 5182A melanoma cells were injected subcutaneously into the flank of female CB17 SCID mice (Charles River Laboratories, Wilmington, Massachusetts). Twelve days after injection when tumors > 10 mm in diameter had formed, animals were randomized in groups. They received either 10 mg per kg clusterin-2'MOE AS-ODN, 10 mg per kg scrambled oligonucleotide in 200 μL of 0.9% saline, or saline alone by i.p. injection. Animals were injected daily from day 12 to 15 and every third day thereafter until the end of the experiment. On day 23, tumors from each group were harvested for analysis of clusterin expression. On days 23–26, animals received daily i.p. injections of dacarbazine at a concentration of 80 mg per kg. Tumor size was measured by a caliper and tumor volume was calculated by the formula: [(largest diameter in mm) × (smallest diameter in mm)^2] × π/6.

Treatment groups consisted of 14 animals in the antisense- and scrambled-oligonucleotide groups and of seven animals in the saline groups. The protocol was approved by the local animal welfare committee.

All experiments except the in vivo experiment were independently performed at least three times.

We are indebted to Ms S Strommer (Department of Clinical Pharmacology, University of Vienna), Dr R Kunstfeld, and Dr R Loewe for their helpful discussion of the manuscript.

DOI: 10.1111/j.0022-202X.2005.23720.x

Manuscript received September 5, 2004; revised November 11, 2004; accepted for publication December 16, 2004

Address correspondence to: Dr Christoph Hoeller, Department of Dermatology, University of Vienna, Waehringerguertel 18-20, 1090 Vienna, Austria. Email: christoph.hoeller@univie.ac.at

References


