

The human IgM antibody SAM-6 induces tumor-specific apoptosis with oxidized low-density lipoprotein

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Abstract

Lipids are essential for normal and malignant cells during growth and differentiation. The turnover is strictly regulated because an uncontrolled uptake and accumulation is cytotoxic and can lead to lipoapoptosis: lipoptosis. The human monoclonal antibody SAM-6 binds to a cell surface receptor on malignant cells and to oxidized low-density lipoprotein (LDL). SAM-6 induces an excess of intracellular lipids, by overfeeding malignant cells with oxidized LDL, via a receptor-mediated endocytosis. The treated cells overaccumulate depots of cholesteryl esters and triglycerides. This lipid overaccumulation is tumor specific; non-malignant cells neither bind the antibody nor harvest lipids after incubation. Because for both forms of apoptosis, the death domain dependent ("extrinsic") and independent ("intrinsic"), the activation of proteases is crucial, we also investigated this pathway in more detail. It was found that shortly after internalization of antibody/oxidized LDL/receptor complex and formation of lipid depots, cytochrome *c* is released by mitochondria. Followed by this, initiator caspase-8 and caspase-9 and effector caspase-3 and caspase-6 are activated. The mechanism of mitochondrial trigger (e.g., by free fatty acids) is under investigation. However, the present data indicate that the SAM-6 antibody induces an intrinsic-like form of apoptosis by overfeeding malignant cells with lipoproteins. [Mol Cancer Ther 2007;6(1):326–33]

Introduction

The mission of immunity is to discriminate between "self" and "nonself" and to spare the first and to eliminate the

last. Immunity in vertebrates, with exception of cartilaginous fish, can be divided into a primary (innate) and a secondary (acquired) immune response (1, 2). The innate immunity is the first line of defense and the stimulus for the secondary immunity and for immunologic memory (3). It is equipped with natural killer cells, dendritic and mast cells, $\gamma\delta$ T cells, macrophages, and natural IgM antibodies, produced by B1 cells in mice and CD5⁺ lymphocytes in humans (4–10). The targets are exogenous "nonself" particles, like bacteria, viruses, and fungi, but innate immunity also takes care about endogenous problems, like modified and secreted molecules and transformed cells (2, 11–14).

The innate response is invariable and works by using a transmitted germ line-coded pool of specific receptors (15–17). These receptors belong to a recently discovered family of nonclonally expressed pattern recognition receptors that show homology with the *Drosophila* Toll protein and the human interleukin-1 receptor family (18). These Toll-like receptors do not recognize specific single structures, but specific patterns (termed pathogen-associated molecular patterns), and can be categorized into those that signal and those that are secreted (14). These specific patterns are conservative structures like carbohydrates on glycoproteins and glycolipids and repetitive structures (e.g., lipopolysaccharide; refs. 15, 18, 19), and they are expressed independently from mutational events (15).

In particular, natural IgM antibodies play an important role in primary defense mechanisms (5, 8, 20–23). They are known to be involved in early recognition and elimination of external invaders like bacteria and viruses, cellular waste, and modified self (24–26). In addition, sufficient evidence has been collected to show that natural IgM antibodies are also involved in the immune surveillance mechanisms against precancerous and cancerous cells (13, 21, 23, 27–29).

The human natural monoclonal IgM antibody SAM-6 was isolated from a stomach cancer patient and induces lipoptosis, an apoptotic lipid concentration in malignant cells. The antibody binds to a membrane molecule specifically expressed on cancer cells (30). We show in this article that the SAM-6 also binds to oxidized low-density lipoprotein (LDL), and that it carries oxidized LDL into the cell via a receptor-mediated endocytosis followed by an intrinsic form of apoptosis *in vitro* and *in vivo*.

Materials and Methods

Cell Culture

The SAM-6-producing hybridoma cell line was generated and cultured as described previously (30). Stomach

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carcinoma cell line 23132/87 and pancreatic carcinoma cell line BXPC-3 were cultured in RPMI 1640 (PAA, Vienna, Austria) supplemented with 10% FCS, 2 mmol/L glutamine, and penicillin/streptomycin (both 1%). The cells were incubated in a humidified 5% CO₂ atmosphere at 37°C.

SAM-6 Purification

SAM-6-producing hybridoma cells were grown in AIM/V serum-free medium (Invitrogen, Karlsruhe, Germany) in a miniPerm bioreactor (VIVASCIENCE, Hannover, Germany). For purification of IgM, supernatants were purified via an ion-exchange column (HiTrap SP FF column, Amersham Bioscience Europe, Freiburg, Germany) using a fast protein liquid chromatography system. The antibody was dissolved in PBS and stored at -70°C. Purity of the antibody was determined on SDS-gel electrophoresis, and activity was determined immunohistochemically and functionally.

LDL and Oxidized LDL Preparations

The LDL used in all assays was obtained from Sigma (Taufkirchen, Germany). Native LDL was purchased from the Medical University Clinic, Würzburg (kindly provided by Dr. J. Galle). To prepare oxidized LDL, the native LDL was oxidized with 20 µmol/L CuSO₄ for 3 or 15 h at 37°C. To determine the amounts of oxidized LDL, a specific enzyme immunoassay was done (Oxidized LDL ELISA, Mercodia, Uppsala, Sweden) following the supplier's manual.

ELISA

ELISA was done using standard protocols. Briefly, flexible, flat-bottomed 96-well plates (Becton Dickinson Labware Europe, Heidelberg, Germany) were incubated with 10 µg/mL LDL, high-density lipoprotein (Sigma), or Cu²⁺-oxidized LDL and incubated with 60 µg/mL SAM-6 antibody or SAM-6 antibody in the indicated concentrations. Plates were then incubated with horseradish peroxidase-coupled secondary antibody (rabbit anti-human IgM, DAKO, Hamburg, Germany) followed by incubation with OPD (DakoCytomation, Glostrup, Denmark) and measurement at 490 nm in an ELISA reader.

Apoptosis Assay

The extent of antibody-induced apoptosis on human pancreatic carcinoma cells (BXPC-3) was analyzed by the Cell Death Detection ELISA^{PLUS} kit (Roche, Mannheim, Germany) following the supplier's manual. Briefly, 1 × 10⁴ tumor cells were incubated with SAM-6 antibody in the presence of 20 µmol/L differently strong Cu-oxidized LDL for 48 h at 37°C. Unrelated human IgM (Chrompure IgM, Dianova, Hamburg, Germany) served as the negative control. After cell lysis, supernatants were transferred into a streptavidin-coated microtiter plate, the immunoreagent was added (mixture of 10% anti-histone-biotin, 10% anti-DNA POD, and 80% incubation buffer), and incubated for 2 h at room temperature on a microtiter plate shaker at 250 rpm. POD was determined photometrically with an ABTS substrate. The antibody-induced apoptosis was measured at 405 nm against ABTS solution as a blank (reference wavelength ~490 nm).

TLC

Human pancreatic cancer cells (BXPC-3) cultured in medium supplemented with LDL were incubated with 100 µg/mL antibody SAM-6 or human unrelated control IgM in the same concentration for 48 h. Cells were harvested, washed twice with PBS, and homogenized, and intracellular lipids were extracted. The lower organic phase was dried under N₂, and the extracted lipids were solved in 150 µL chloroform/methanol (2:1), and 25 µL of this solution were loaded on the thin layer plate (coated with silica gel). The TLC was done with a solvent system of hexane/ethyl acetate/acetic acid (90:10:1).

Analysis of Apoptosis by Hypotonic Propidium Iodide Staining

Pancreatic carcinoma cells (BXPC-3; 2 × 10⁵) were incubated with SAM-6 supernatant (10 µg/mL) or isotype-matched control antibody (Chrompure IgM, Dianova) in the same concentration over a period of 120 h. In a cycle of 24 h, adherent cells were trypsinized, pooled with suspended cells in the cell culture supernatant, and washed once with cold PBS (pH 7.4). After centrifugation, the cell pellet was resuspended in 700 µL cold and freshly prepared lysis buffer containing 0.1% Triton X-100, 0.1% sodium citrate, 100 µg/mL RNase, and 50 µg/mL propidium iodide.

After 5 min, DNA content was detected by flow cytometry and analyzed using WinMDI software.

Labeling of SAM-6 Antibody with FITC

For immunofluorescence studies, conjugation of monoclonal antibody SAM-6 and isotype control IgM (Chrompure IgM, Dianova) was done with Fluoro Tag FITC Conjugation kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol.

SAM-6 Endocytosis

Endocytosis was determined for SAM-6 antibody on human pancreatic carcinoma cell line BXPC-3. Conjugated antibodies at a final concentration of 40 µg/mL were directly given to 1 × 10⁶ cells and incubated for 30, 60, and 120 min at 37°C. Cells were harvested, rinsed, and resuspended in PBS (pH 7.4). One hundred microliters of each cell suspension were fixed on slides. Finally, the slides were mounted with Fluorescent Mounting Medium (Dako-Cytomation, Carpinteria, CA) and analyzed by confocal microscopy.

Cytochrome *c* Assay

To analyze whether cytochrome *c* was released during SAM-6-induced apoptosis, the Cytochrome *c* ELISA kit (Calbiochem, La Jolla, CA) was used. In short, 1.5 × 10⁶ stomach carcinoma cells (23132/87) were incubated with 200 µg/mL purified SAM-6 or unrelated IgM antibody for 1 and 4 h, respectively. After trypsinization, the cells were washed thrice with cold PBS, resuspended in lysis buffer, and incubated for 1 h at room temperature with gentle mixing. After centrifugation, supernatants were diluted (1:10) with Calibrator Diluent RD5P (1×), and a mixture (1:1) of each of the diluted samples was added into the microtiter plate delivered in the kit. After an incubation for 2 h, the cytochrome *c* conjugate was added into each well

and incubated for another 2 h. After the addition of Substrate Solution (1:1 mixture of Color Reagent A and B) to each well, the plate was finally incubated for 30 min at room temperature, and cytochrome *c* release was determined photometrically at 415 nm (reference wavelength = 540 nm) after the addition of Stop Solution.

Caspase Assay

For screening SAM-6 antibody-treated cells for caspase-2, caspase-3, caspase-6, caspase-8, and caspase-9 activity, the Apo Target Colorimetric Protease Assay Sampler kit (Calbiochem) was used following the suppliers manual. In short, 3×10^6 stomach carcinoma cells (23132/87) were incubated with 200 $\mu\text{g}/\text{mL}$ purified SAM-6 or unrelated IgM antibody for 1 and 4 h, respectively. After trypsinization, the cells were resuspended in cold lysis buffer. They were incubated for 10 min on ice and centrifuged for 1 min at $10,000 \times g$. To determine the amount of protein in the cell lysates, Bradford assay was applied. Each cytosol extract was diluted to a protein concentration of 4 $\mu\text{g}/\text{mL}$. Then reaction buffer containing DTT and the various conjugated protease substrates were added to the samples in a 96-well microtiter plate. A mixture (1:1) of lysis and reaction buffer served as a blank. After incubation for 2 h at 37°C and 7% CO_2 in a humidified CO_2 incubator, the absorption and thus the extent of caspase activity was measured in an ELISA reader at 415 nm. For experiments with caspase-3 inhibitor, the Caspase-3 Cellular Activity Assay kit (Calbiochem) was used following the supplier's manual, using similar conditions as described above.

In vivo Experiments

To determine the effects of antibody SAM-6 on tumor cell growth *in vivo*, a nude mouse/human stomach carcinoma cell system was used. Briefly, 2×10^6 stomach carcinoma cells (23132/87) were injected s.c. into 6- to 7-week-old NMRI *nu/nu* mice (Harlan Winkelmann GmbH, Borcheln, Germany) followed by injections of antibody SAM-6 when tumors reached visible size; 50, 250, or 500 μg antibody was given, respectively, at days 9, 11, 14, 16, and 17 i.p. after carcinoma cell injection. Control mice were injected with unrelated human IgM (Chrompure IgM, Dianova). The experiment was terminated when tumors had reached maximal tolerable size (day 18), whereupon the mice were sacrificed, tumor volume was determined, and organs and tissues were inspected for the spread of tumors and other alterations. Statistical significance between control group and SAM-6-treated animals was assayed using Student's *t* test; 95% confidence interval ($P < 0.05$) was considered significant.

Results

DNA Degradation in SAM-6-Induced Apoptosis

DNA degradation is a good indicator for apoptotic activity. Propidium iodide integrates into double-stranded DNA and is therefore used to represent the DNA content of a cell. Cells in the G_1 phase of the cell cycle have a single diploid chromosome set (2n), whereas cells in G_2 have a double diploid chromosome set (4n). During apoptosis, the

DNA is degraded by different enzymes resulting in DNA fragments smaller than 2n. These sub- G_1 fragments are specific for apoptotic events and could be illustrated by flow cytometry.

To investigate this activity for SAM-6, pancreatic cancer cells were treated with SAM-6 antibody or control IgM. Every 24 h, cells were harvested, propidium iodide was added, and DNA content was analyzed by flow cytometry (Fig. 1). Due to the low sensitivity of the method, it took several days to visualize, by fluorescence-activated cell sorting analysis, the accumulation of degraded DNA, but Fig. 1D clearly shows that SAM-6 treatment results in an increase of "apoptotic" DNA.

SAM-6 *In vivo* Activity

To determine the effects of antibody SAM-6 on tumor cell growth *in vivo*, a nude mouse/human stomach carcinoma cell system was used. A concentration of 2×10^6 cells derived from the human stomach carcinoma cell line 23132/87 were injected i.p. into NMRI *nu/nu* mice. Nine days after the inoculation of tumor cells, different doses of SAM-6 antibody were injected i.p. Unrelated human control IgM and NaCl solution (0.9%) served as negative controls. The antibody was given again on days 11, 14, 16, and 17 after carcinoma cell implantation. Throughout the duration of the study, tumor growth was controlled macroscopically. After 18 days, the mice were sacrificed, tumor volumes were determined, and Student's *t* test was used to compare tumor sizes between the treatment and control groups. The tumors that developed during the course of the experiment showed a significant reduction in volume when treated with SAM-6 antibody (Fig. 2). Moreover, the reduction of tumor volume in mice treated with antibody SAM-6 is dose dependent. Mice already treated with 50 μg SAM-6 antibody show a clearly reduced tumor volume compared with the control groups. Animals treated with 250 and 500 μg SAM-6 antibody have statistical significantly smaller tumor volumes (*t* test, $P < 0.005$ for both concentrations) when compared with the IgM control.

SAM-6-Induced Lipid Uptake in Cancer Cells

Antibody SAM-6 induces the accumulation of lipids in tumor cells after binding to the SAM-6 membrane receptor, as published recently (30). This effect is tumor specific; SAM-6 does not bind to or kill nontransformed epithelial or fibroblastic cells. To investigate the intracellular lipids accumulated in cancer cells after SAM-6 treatment, a TLC was prepared. Pancreatic cancer cells cultured in medium supplemented with LDL were incubated with antibody SAM-6 or human unrelated control IgM for 48 h. Cells were harvested and homogenized and intracellular lipids were isolated. Then lipids were separated using TLC. As shown in Fig. 3, cancer cells treated with antibody SAM-6 showed an elevated level of intracellular cholesteryl ester, triglycerides, and, to a lower extent, cholesterol compared with cells treated with human unrelated control IgM. As cholesteryl ester and triglycerides are the main lipid components of lipoproteins, these results indicate that during SAM-6 treatment, LDL is transported into cancer cells and accumulated up to a deadly concentration.

SAM-6 Binding to LDL/Oxidized LDL

LDL is very sensitive for oxidation and exists *in vivo* in a native form and an oxidized form. Oxidation goes along with a modification of the molecule and makes it a target

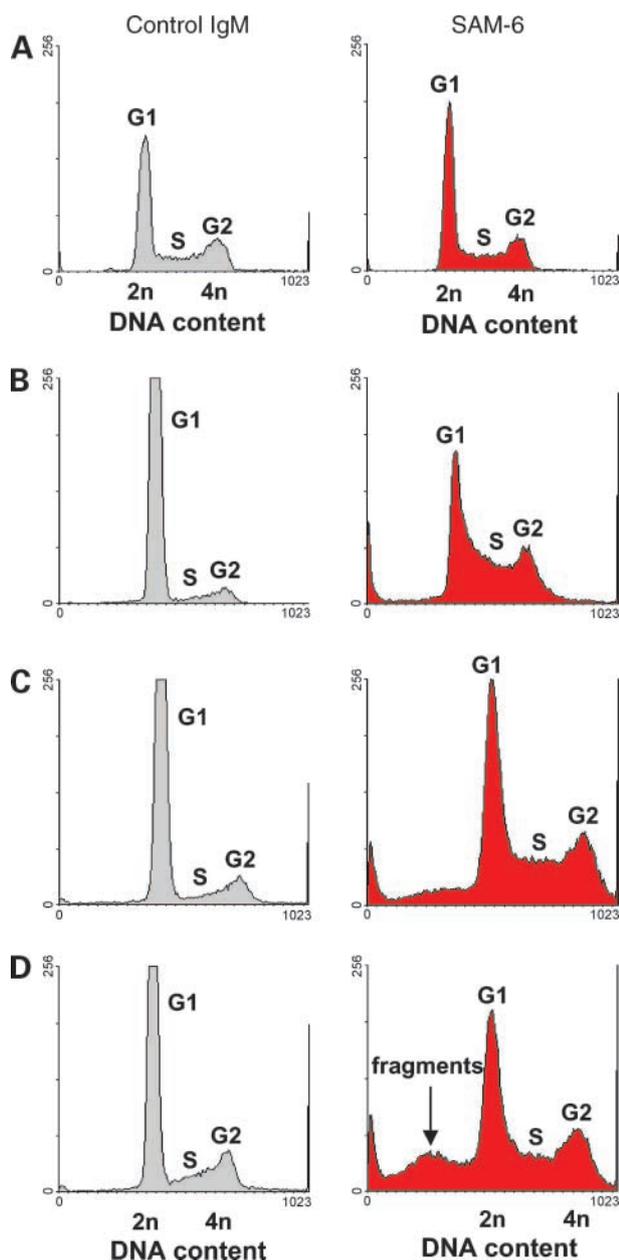


Figure 1. Fluorescence-activated cell sorting analysis of SAM-6–induced apoptosis by hypotonic propidium iodide staining. Fluorescence-activated cell sorting analysis of propidium iodide–stained DNA from pancreatic cancer cell line BXP-3 treated with SAM-6 antibody or control IgM over a period of 5 d. Every 24 h, cells were harvested and incubated with propidium iodide. After 5 min, DNA content was detected by flow cytometry and analyzed using WinMDI software. **A**, control, unincubated cells. **B**, incubation with SAM-6 antibody or control IgM for 3 d. **C**, incubation with SAM-6 antibody or control IgM for 4 d. **D**, incubation with SAM-6 antibody or control IgM for 5 d. SAM-6 treatment results in an increase of degraded apoptotic DNA.

for immune responses. To investigate which form is detected by the antibody SAM-6, ELISA binding assays were prepared using freshly prepared native LDL (weakly oxidized), Cu-oxidized LDL, and high-density lipoprotein. Compared with the isotype-matched human control antibody, antibody SAM-6 shows binding to freshly prepared LDL and to Cu-oxidized LDL but not to high-density lipoprotein (Fig. 4A). To strengthen the specificity of antibody binding to oxidized LDL, pancreatic carcinoma cells were incubated with antibody SAM-6 in the presence of differently strong oxidized lipoprotein, followed by performance of an apoptosis assay. Figure 4B shows that the SAM-6–induced apoptosis of tumor cells could be enhanced by prolonged Cu^{2+} -mediated oxidation of LDL.

SAM-6 Endocytosis

The antibody SAM-6 binds to cell membrane antigen of 140 kDa (30). This antibody/receptor binding initiates the accumulation of lipids and the apoptotic cascade. SAM-6 also binds to oxidized LDL and carries this into cancer cells. Lipoproteins are normally internalized by a receptor-mediated endocytosis. To investigate what happens after SAM-6 binding to a cancer cell membrane, SAM-6 and an isotype control were conjugated with FITC. Conjugated antibodies in the presence of LDL were directly given to human pancreatic carcinoma cell-line BXP-3, and incubated for 30, 60, and 120 min. Cells were finally analyzed by confocal microscopy. After 30 min of incubation with antibody SAM-6, antibody binding to the cell surface could be observed (Fig. 5A). After 60 min, the antibody is concentrated at the membrane, seen as a typical formation of “capping” (Fig. 5B). One hour later, the antibody is completely internalized into the cell (Fig. 5C). In comparison, the labeled control antibody do not show similar events (Fig. 5D–F). It can be assumed that the oxidized LDL is carried into the cell together with the antibody.

SAM-6 Apoptotic Pathway

With regard to SAM-6–induced apoptosis, its explanation almost certainly lays in the disturbed lipid homeostasis. Thus far, however, nothing was known about the pathway among antibody binding, lipid accumulation, and the ultimate cell death just as little as about, for example, which caspases are activated. A better understanding of the signaling pathway activated by SAM-6 is essential to come closer to the aim, making a further contribution to innovative therapies for the fight against cancer. Therefore, we have investigated which caspases are involved in the SAM-6–induced apoptotic process, and whether this pathway is completely exclusive or probably resembles one of the two known pathways (“intrinsic/extrinsic”).

To examine whether cytochrome *c* was set free in the gastric cancer cells after incubation with SAM-6 and the control IgM, respectively, the cytochrome *c* ELISA kit was applied. The sandwich enzyme immunoassay technique proved that cytochrome *c* was released at a higher level in the cells treated with the monoclonal antibody SAM-6 than in the cells treated with the unrelated human IgM after 1 h. Additionally, the amount of the polypeptide in both samples differed more than after 4 h of incubation. Also

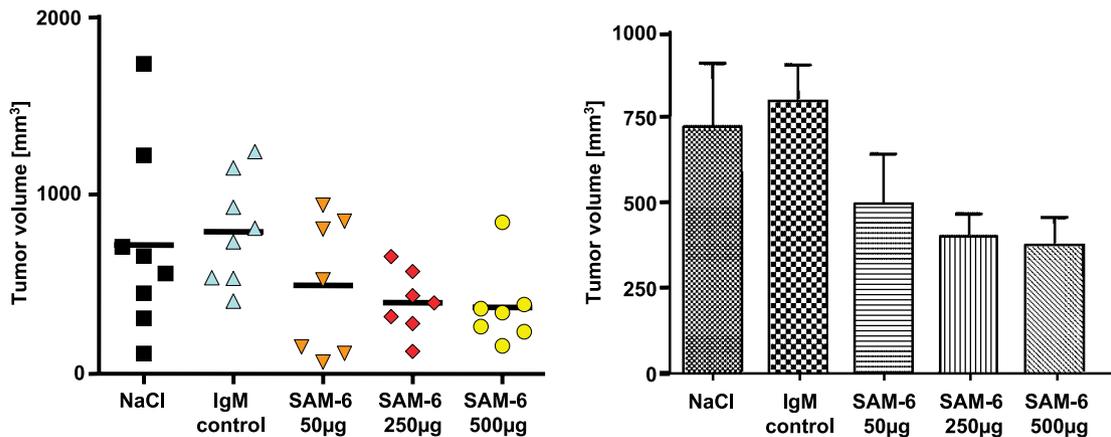


Figure 2. SAM-6 – induced tumor cell apoptosis *in vivo*. Stomach carcinoma cells (23132/87; 2×10^6) were injected s.c. into 6- to 7-wk-old NMRI *nu/nu* mice (Harlan Winkelmann) followed by injections of antibody SAM-6 when tumors reached visible size; 50, 250, or 500 µg antibody was given, respectively, at days 9, 11, 14, 16, and 17 i.p. after carcinoma cell injection. Control mice were injected with unrelated human IgM (Chrompure IgM, Dianova). The experiment was terminated when tumors had reached maximal tolerable size (day 18), whereupon the mice were sacrificed, and tumor volume was determined. SAM-6 significantly reduces tumor volume in a dose-dependent manner.

observable is the decrease in the SAM-6 sample after 4 h (Fig. 6A). This clearly indicates that in the SAM-6–induced pathway, a perturbation of the mitochondria occurred, leading to the break of the outer membrane and resulting in the cytochrome *c* release. The cytochrome *c* level in SAM-6–treated cells came close to that of the control after 4 h of incubation, signifying that the mitochondrial breakdown only occurred at an early stadium of the pathway.

To investigate which caspases and whether caspases were induced at all by SAM-6, the Apo Target Colorimetric Protease Assay Sampler kit was used. After a 1-h incubation with the antibodies, the activity of caspase-2, caspase-3, caspase-6, caspase-8, and caspase-9 was measured. The initiator caspase-8 and caspase-9 but not caspase-2 (data not shown) were activated in the cells treated with SAM-6 compared with those treated with the unrelated human IgM control already after 1 h (Fig. 6B). In addition, after 4 h, an activation of the effector caspase-3 and caspase-6 could be noticed.

To exclude artifacts, an experiment using a caspase-3 inhibitor was prepared additionally as an example. Figure 6C shows the suppressed caspase-3 activity when the inhibitor was added. In absence of the caspase-3 inhibitor, a clear activation of caspase-3 could be observed in the SAM-6–treated tumor cells.

Discussion

Some metabolic mechanisms of lipoapoptosis have been described, and some causes are discussed, but reagents, which directly induce these syndromes, have thus far not been identified. The human monoclonal antibody SAM-6 inhibits tumor growth *in vitro* and *in vivo* by feeding cancer cells with oxidized LDL and inducing a deadly accumulation of cholesterol, cholesteryl ester, and triglycerides. Shortly after receptor-mediated endocytosis of the antibody/LDL complex, a release of cytochrome *c* from the

mitochondria triggers the initiator caspase-8 and caspase-9. This is followed by the effector caspase-3 and caspase-6 and lipoptosis, the apoptotic cell death. The antibody SAM-6 is the first defined protein that disturbs the lipid metabolism of a cancer cell by inducing an extensive lipid storage and an intrinsic pathway of apoptosis.

The human monoclonal IgM antibody SAM-6 was isolated from stomach cancer patient by using the human

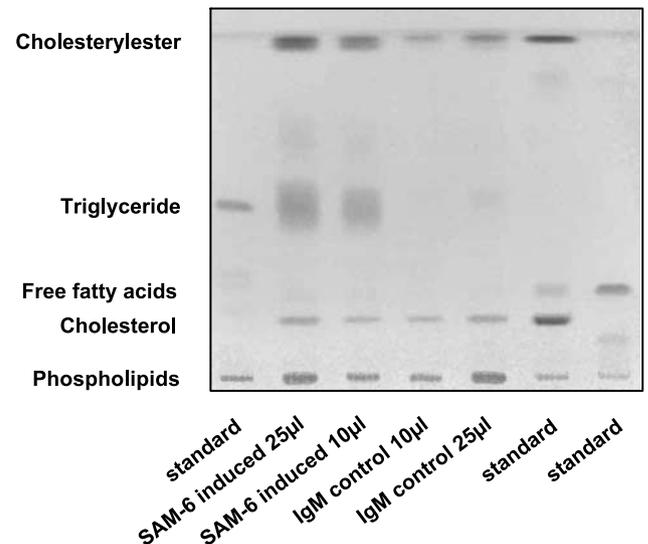


Figure 3. Chromatographic analysis of SAM-6–induced accumulation of intracellular lipids in tumor cells. Human pancreatic carcinoma cells (BXP-3) cultured in medium supplemented with LDL were incubated with antibody SAM-6 or human unrelated control IgM for 48 h. Cells were harvested and homogenized, and intracellular lipids were isolated. The lipids were separated using TLC. Tumor cells treated with antibody SAM-6 show an elevated level of intracellular cholesteryl ester and triglycerides and, to a lower extent, cholesterol compared with tumor cells treated with human unrelated control IgM.

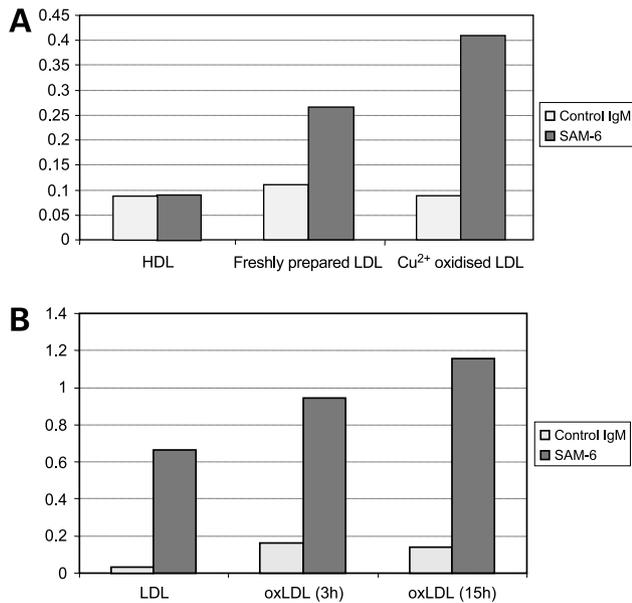


Figure 4. ELISA binding studies of human antibody SAM-6 to lipoproteins and apoptosis assay. ELISA plates were preincubated with 10 $\mu\text{g/mL}$ LDL, high-density lipoprotein (HDL), or oxidized LDL (oxLDL), respectively, overnight, followed by incubation with human monoclonal IgM antibody SAM-6 or human isotype-matched control IgM. **A**, antibody SAM-6 shows binding to LDL compared with the isotype-matched human control antibody. In contrast, antibody SAM-6 shows no binding to high-density lipoprotein. Antibody SAM-6 shows an increased binding after Cu^{2+} oxidation of LDL. **B**, antibody-induced apoptosis of tumor cells in the presence of differently strong Cu-oxidized LDL was measured by Cell Death Detection ELISA^{PLUS}. Pancreatic carcinoma cell line BXP-3 were incubated with antibody SAM-6 and unrelated human IgM isotype control in a concentration of 50 $\mu\text{g/mL}$ for 48 h in the presence of LDL or Cu-oxidized LDL (Cu oxidation for 3 h or 15 h, respectively). Amounts of apoptotic cells were determined photometrically at 415 nm and reference wave length of 490 nm. SAM-6 apoptosis is enhanced in the presence of increased Cu^{2+} -oxidized LDL.

TRIOMA technology. The antibody is secreted as a pentamer with a lambda light chain. It is germ line coded and is most likely part of the natural immunity (30). In binding and functional studies, it was found that the SAM-6 reactivity is restricted to malignant tissue (30). The binding of SAM-6 could be removed by glycosidase treatment of the target cell, indicating a carbohydrate epitope of the antibody (data not shown). This expression of posttranscriptionally modified carbo epitopes on malignant seems to be a common feature for malignant cells and was already proven for a series of other tumor-specific human monoclonal IgM antibodies (23, 29, 31, 32).

The antibody SAM-6 increases the intracellular lipid content of malignant cells but not of normal cells. This specific overaccumulation of cholesteryl esters and triglycerides induces an apoptotic cell death (30). Lipids and their metabolites play an important role in cellular differentiation and growth (33). A balanced lipid metabolism is crucial for all cells, and extensive lipid storage can result in cell death. This was shown in several animal studies and was also described for some inherited and acquired human diseases (33). When lipids overaccumulate

in non-adipose tissue due to overnutrition, fatty acids enter deleterious pathways, such as ceramide production, and can induce apoptosis (33). It was shown in mice and rats that lipotoxic cardiomyopathy is caused by accumulation of cardiotoxic lipids, which can induce the death of cardiac monocytes (34). Similar data on heart failure induced by lipid accumulation were obtained for humans by analyzing postmortem samples (35).

Lipoproteins carry the lipids needed for the production of cell membranes and hormones to every cell in the body. High levels of LDL, caused, for example, by liver dysfunction or by nutritional excesses, have been shown to result in atherosclerotic plaques that are believed to be a risk factor for cardiovascular diseases, the main cause of death in Western countries (36). In addition, LDL is very sensitive to lipid peroxidation mediated by radicals, with oxidized LDL being produced as a result (37). Although modified LDL represents only a small portion of circulating LDL (<10%), its involvement in the pathogenesis of atherosclerosis is generally accepted (38–40).

The immunogenicity of oxidized LDL is very high, and antibodies against oxidized LDL can be detected in almost all humans regardless of age (41). Oxidation of LDL often result in the formation and expression of large numbers of neoepitopes and makes it a target for innate immunity (42–44). Specific pattern recognition receptors, like scavenger receptors on macrophages, bind and remove modified oxidized LDL from the circulation. Another consequence of the modification is the production of antibodies, which have been found in humans, rabbits, and mice (45). Most of these antibodies are part of the innate immunity, and their biological function is the clearance of modified molecules, like oxidized LDL, to prevent cellular and molecular damage by oxidation. It has been found that some of these antibodies are affinity matured auto antibodies, participating in several diseases (46), whereas others are involved in a protective process and are weapons of innate immune mechanisms (47, 48).

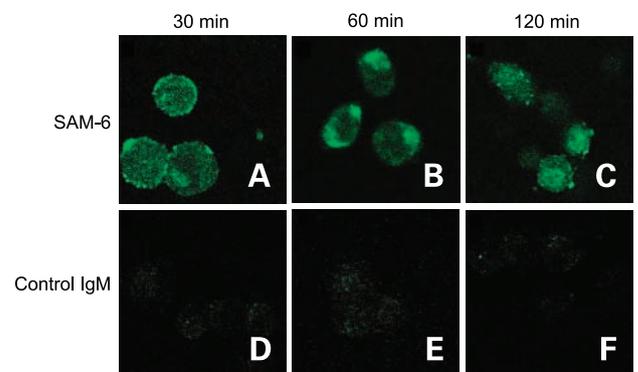


Figure 5. Immunofluorescence of SAM-6 endocytosis. Pancreatic carcinoma cells BXP-3 were incubated with fluorochrome-labeled SAM-6 antibody or unrelated human control IgM. After 30, 60, and 120 min, cells were exposed on slides, fixed, and analyzed using confocal microscopy. **A**, 30-min antibody binding. **B**, 60-min "capping". **C**, antibody SAM-6 is completely internalized into the cell. In comparison, the labeled control antibody does not show similar events (**D–F**).

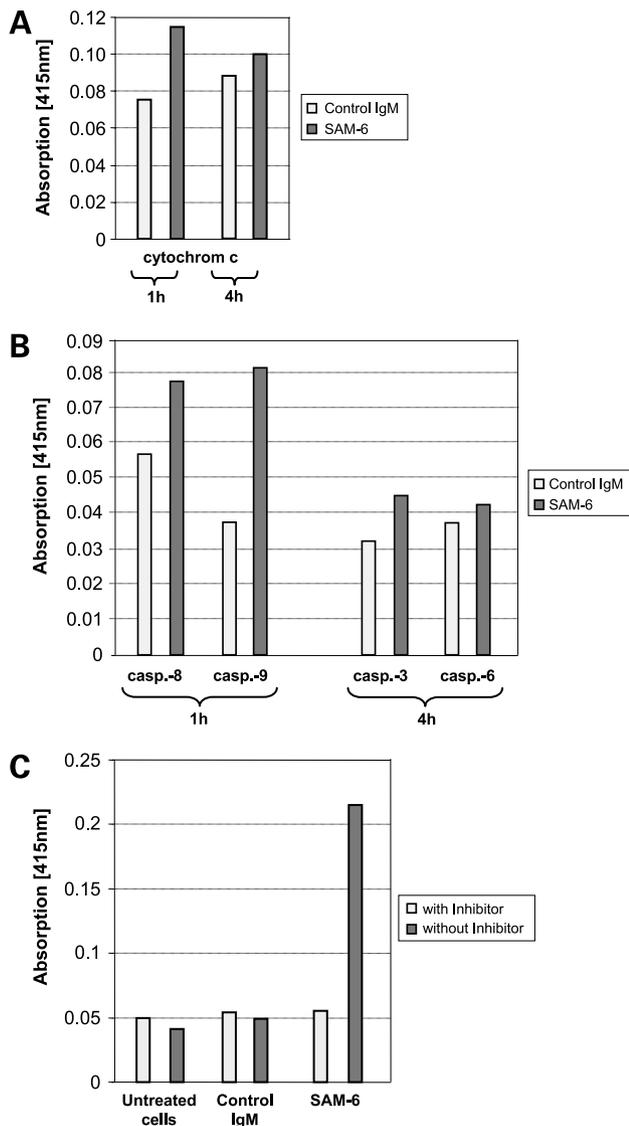


Figure 6. Analysis of SAM-6-induced apoptosis by measurement of cytochrome *c* release and activation of caspase-8, caspase-9, caspase-3, and caspase-6. A Cytochrome *c* ELISA was used to analyze the cytochrome *c* release in SAM-6-induced apoptosis. Gastric cancer cells (23132/87) were incubated with antibody SAM-6 or unrelated human IgM (both in 200 μ g/mL) for 1 and 4 h, respectively, and tested for cytochrome *c* release. **A**, in SAM-6-treated tumor cells, the cytochrome *c* release is higher than with unrelated, human control IgM after 1 and 4 h. The Apo Target Colorimetric Protease Assay was used to investigate the activities of caspase-2, caspase-3, caspase-6, caspase-8, and caspase-9 in tumor cell line 23132/87 after incubation with antibody SAM-6 or unrelated human IgM (concentration of both: 200 μ g/mL) for 1 or 4 h. **B**, after 1 h, caspase-8 and caspase-9 were activated in the cells treated with SAM-6 antibody, and after 4 h, an activation of caspase-3 and caspase-6 could be noticed. **C**, suppressed caspase-3 activity could be shown when a caspase-3 inhibitor was added. In the absence of the caspase-3 inhibitor, a clear activation of caspase-3 could be observed in the SAM-6-treated tumor cells.

A good example is the natural IgM antibody EO6, described by Palinski et al. (49, 50). It was cloned from the spleens of hypercholesterolemic apolipoprotein E-deficient and binds to oxidized LDL. The antigenic

structure was characterized as a phospholipid containing the phosphorylcholine head group, such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine, which is a major oxidized lipid of oxidized LDL and also present on microbes (51). Interestingly, the antibody also binds to the same phosphorylcholine moiety on apoptotic thymocyte cells, which are known to show enhanced expression of oxidized biologically active phospholipids (51). Furthermore, the EO6 antibody blocks the uptake of oxidized LDL to macrophage scavenger receptors and might therefore be useful for prevention of atherosclerosis (50, 52). Both natural antibodies SAM-6 and EO6 bind to oxidized LDL and to a neoepitope on malignant or apoptotic thymocytes, respectively. The cellular epitope most likely resembles the one on oxidized LDL (50). It is likely that they bind to different epitopes on oxidized LDL because EO6 cross-reacts with apoptotic thymocytes, whereas SAM-6 does not only bind to malignant cells but also induces apoptosis (30).

However, their epitopes have one thing in common: they are "foreign" to an intact organism. In addition, the data show that innate immunity uses receptors that are limited in number, and, of necessity, these are focused on highly conserved structures found in many "nonself" particles. These kind of cross-reacting natural antibodies are good examples for nature's economic fantasy in immune surveillance processes.

Acknowledgments

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