Lipoptosis: Tumor-Specific Cell Death by Antibody-Induced Intracellular Lipid Accumulation

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ABSTRACT

A balanced lipid metabolism is crucial for all cells. Disturbance of this homeostasis by nonphysiological intracellular accumulation of fatty acids can result in apoptosis. This was proven in animal studies and was correlated to some human diseases, like lipotoxic cardiomyopathy. Some metabolic mechanisms of lipo-apoptosis were described, and some causes were discussed, but reagents, which directly induce lipo-apoptosis, have thus far not been identified. The human monoclonal IgM antibody SAM-6 was isolated from a stomach cancer patient by using the conventional human hybridoma technology (trioma technique). The addition of SAM-6 to tumor cells leads to an increase in the intracellular accumulation of neutral lipids, followed by tumor cell apoptosis. The antibody SAM-6 does not react with noncancerous human epithelial and fibroblastic cells, because the $M_f$, 140,000 membrane molecule, recognized by the antibody, is specifically expressed on human malignant cells. The antibody is coded by the germ-line genes IgHV3-30.3*01 and IgLV3-1*01 and is a component of the intact immune system to cancer. In this article, we describe an antibody-induced tumor-specific cell death, named lipoptosis. This is, to our knowledge, the first description of this specific form of lipo-apoptosis as an antibody-mediated mechanism of tumor cell killing.

INTRODUCTION

Fatty acids and their metabolites play an important role in cell differentiation (1, 2). Extensive lipid storage can result in apoptotic cell death. This was shown in several animal studies and was also described for some inherited and acquired human diseases (2, 3). When lipids over-accumulate in nonadipose tissue due to over-nutrition, fatty acids enter deleterious pathways such as ceramide production and can cause apoptosis (2). It was shown in mice and rats that lipotoxic cardiomyopathy is caused by accumulation of cardio-toxic lipids, which can induce the death of cardiac monocytes (4, 5). Similar data on heart failure induced by lipid accumulation were obtained for humans by analyzing postmortem samples (6, 7). The interferences with the lipid content in tumor cells by antibodies might be a novel strategy of cancer therapy. The human monoclonal IgM antibody SAM-6 was isolated from a stomach cancer patient by using the conventional human hybridoma technology (trioma technique; Refs. 8, 9). This technology offers not only an optimal approach to study the humoral immunity of humans with tumor systems (8, 18, 19). Clinical studies of SC-1 showed that regression and apoptosis of primary stomach cancers can be induced without any detected toxic cross-reactivity to normal tissue (22).

The fully human monoclonal IgM antibody PAM-1 was isolated from a patient with a stomach carcinoma, too (24). The PAM-1 receptor was found to be a $M_f$, 130,000 integral membrane glycoprotein (15), homologous to cysteine-rich fibroblast growth factor receptor 1 (25). This post-transcriptionally modified receptor is expressed on nearly all epithelial cancers of every type and origin, but not on healthy tissue. It is also present on precursor lesions found in the following: H. pylori-induced gastritis; intestinal metaplasia and dysplasia of the stomach; ulcerative colitis-related dysplasia and adenomas of the colon; Barrett metaplasia and dysplasia of the esophagus; squamous cell metaplasia and dysplasia of the lung; and cervical intraepithelial neoplasia I-III (13, 15). Binding of the PAM-1 antibody to its receptor induces apoptosis in vitro and in vivo, most likely by blocking the cysteine-rich fibroblast growth factor receptor 1 (17).

In this paper, we describe a new mechanism of an antibody-induced apoptosis, namely lipoptosis, due to an intracellular accumulation of lipids.

MATERIALS AND METHODS

Somatic Hybridization. The SAM-6 producing hybridoma cell line was generated and cultured as described previously (8, 9).

Cell Culture. Carcinoma cells lines 23132/87 and BXPC-3 were cultured as described previously (11), and nasal septum squamous cell carcinoma cell line RPMI-2650 (DSMZ ACC287, Braunschweig, Germany) was cultured in RPMI-1640 (PAA, Vienna, Austria) supplemented with 10% FCS, 2 mM glutamine, and penicillin/streptomycin (both 1%). Normal human nasal epithelial cells HNEpC-c (PromoCell GmbH, Heidelberg, Germany) were cultured in a special airway epithelial cell growth medium with SupplementMix (PromoCell). All cells were incubated in a humidified, 5% CO$_2$ atmosphere at 37°C.

Immunohistochemical Staining of Paraffin Sections. Paraffin-embedded human tissues were sectioned (2 μm), deparaffinized, and heated in citric acid (pH 5.5) in a pressure cooker for 5 min. The immunohistochemical staining with antibody SAM-6 (4 μg/ml), with positive control antibodies (anti-cytokeratin 8 antibody or anti-cytokeratin 5/6 antibody; diluted 1:20 with BSA/PBS; Dako, Hamburg, Germany) or unrelated human IgM as a negative control (Chrompure IgM in the same concentration; Dianova, Germany) was performed as described elsewhere (10). The sections were analyzed using light microscopy.

Preparation of Tumor Cell Membrane Extracts and Western Blotting. Isolation of membrane proteins from tumor cells was performed as described previously (16). Reducing SDS-PAGE gels (10%) and Western blotting of membrane proteins were performed using standard protocols. In short, blotted nitrocellulose membranes were blocked, followed by incubation for 1 h with 20 μg of SAM-6 human IgM antibody or unrelated human control IgM. The secondary antibody (peroxidase-coupled rabbit antihuman IgM antibody
1:1000; Dianova) was detected with the SuperSignal chemiluminescence kit from Pierce (Perbio Science Deutschland GmbH, Bonn, Germany).

**Apoptosis Assay.** The Cell Death Detection ELISA PLUS (Roche, Mannheim, Germany) was performed as described previously (11).

**Ultrastructural Studies.** Adherent growing stomach carcinoma cell line 23132/87 was incubated with 10 μg/ml SAM-6 antibody or unrelated human control IgM for the indicated periods of time. Then the slides were fixed with 2.5% glutaraldehyde (electron microscopy) or 6.25% glutaraldehyde in Sørensen buffer (pH 7.4; for raster electron microscopy) and prepared for microscopic analysis. Morphology of cells was investigated with scanning electron microscopy and transmission electron microscopy.

**Sudan III.** For intracellular lipid staining, stomach carcinoma cells 23132/87 and pancreas carcinoma cells BXPC-3 were grown on glass slides. Adherent cells were incubated for 48 h with antibody SAM-6 (30 μg/ml) or human unrelated IgM control antibody in similar concentration. BXPC-3 cells were incubated in addition with an antihuman CD95/Fas antibody CH-11 (1 μg/ml; Immunotech, Marseilles, France) for 48 h. After two washing steps with PBS, cells were fixed for 5 min with 60% isopropanol. Before use, a 60% solution of the Sudan III stock (0.5% Sudan III in 100% isopropanol) was matured overnight, filtered, and added to the fixed cells. After 15 min, cells were washed with distilled H₂O, differentiated in 60% isopropanol, washed again, and then counterstained for 6 min with Mayers Hemalaun. Finally, cells were rinsed with water for 10 min, washed with distilled H₂O, and mounted with glycerol gelatin.

**Nile Red Staining.** Lipid staining with the phenoxazine dye Nile red was performed as described before (26). In brief, stomach carcinoma cells 23132/87 were grown on glass slides, and adherent cells were incubated with SAM-6 antibody (30 μg/ml) for 48 h. Cells were then fixed with 1.5% glutaraldehyde for 5 min, washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and incubated in a 1:200 dilution of Nile Red in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and incubated in a 1:200 dilution of Nile Red in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and incubated in a 1:200 dilution of Nile Red in acetone. After an additional washing step with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, cell nuclei were stained with 4',6-diamidino-2-phenylindole (dilution 1:1000 in water) for 8 min. Cells were then washed again and mounted with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL). Fluorescence analysis was performed with Leica TCS SP2 confocal laser microscope. Polar lipids are stained dark red (543 nm), neutral lipids are stained yellow (488 nm), and cell nuclei appear blue (350 nm).

**SAM-6 Purification.** SAM-6 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 on an ion-exchange column (HiTrap SP FF column; Amersham Bioscience Europe, 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 measured immunohistochemically and functionally.

**Toxicity.** For toxicity experiments, BALB/c mice received i.p. injections of 0.5 and 1 mg of SAM-6 antibody, respectively. Control mice were treated with the same amounts of unrelated human control IgM. At days 2 and 14, mice were sacrificed, organs inspected for toxic changes, and blood samples measured for liver enzymes.

### RESULTS

**Immunohistochemistry.** The specificity of the antibody SAM-6 was initially investigated by immunohistochemistry on a panel of malignant and healthy tissues. As shown in Fig. 1, antibody SAM-6 reacts only with tumor cells; the tissues surrounding the malignant areas are not stained. In Fig. 2, additional staining is shown with healthy tissue, and it can be clearly stated that SAM-6 is binding to a receptor specifically expressed on malignant tissue. These results were verified on a broad panel of malignant and healthy tissues (data not shown).

**Western Blot Analysis and Functional Activity.** To determine the antigen of SAM-6, Western blot experiments with membrane preparations of two different cell lines were performed. As shown in Fig. 3A, the SAM-6 antibody binds to a membrane molecule of about M₅ 140,000. The functional activity of SAM-6 was investigated in a specific apoptosis assay. In Fig. 3B, the results of the apoptosis assay on different carcinoma cell lines and on normal epithelial cells are shown. The experiment clearly showed that antibody SAM-6 induces apoptosis of stomach, pancreas, and nasal septum carcinoma cells. In contrast, normal nasal epithelial cells are not affected by SAM-6 treatment.

In Fig. 3C, the morphological changes of antibody SAM-6-induced...
apoptosis are shown on stomach carcinoma cells and on pancreas carcinoma cells. Untreated tumor cells grow in homogenous monolayers. After treatment with antibody SAM-6, the cells become more spindle-shaped and flat and more polarized with more pronounced cytoplasmic elongations. A loss of cell-cell contacts and adhesion could be observed after 48 h. Decrease in cell number is caused by apoptosis, because apoptotic cells get in the solution as a result of lost adhesion.

Scanning Electron Microscopy. To investigate the apoptotic effects of antibody SAM-6 on tumor cells in more detail, ultrastructural studies were performed. Scanning electron microscopy was used to study the morphological and extracellular apoptotic effects. Stomach carcinoma cells were incubated with SAM-6 and a control antibody for 48 h, and the effects were analyzed at different time points. As shown in Fig. 4, initial morphological changes of SAM-6-treated cells after 2 h include the formation of stress fibers (Fig. 4, D and G) and a slight reduction of cell-cell contacts. After 24 h, drastic morphological changes are observed. Cell-cell contacts are infinitely low (Fig. 4, E), cells are either enlarged or condensed, the nuclei are swelled (Fig. 4, H), and the formation of apoptotic bodies is increased. The most dramatic effects are observed after 48 h. Numerous structural plasma membrane alterations are observed in the apoptotic cells: loss of cellular adhesion; smoothing; shrinkage; and out-pouching of membrane segments are recognized as markers associated with cell injury and death. Most importantly, on the shrunken tumor cells, huge packages of membrane vesicles, apoptotic bodies, are clustered (Fig. 4, F). The impressive formation of smooth-surface apoptotic bodies, as shown at the higher magnification, is due to the fact that in contrast to the in vivo recycling by phagocytic cells, in vitro the membrane vesicles remain sitting on the dead cells (Fig. 4).

Transmission Electron Microscopy. To investigate the intracellular apoptotic effects, we performed transmission electron microscopic studies with SAM-6 on stomach carcinoma cells. After 24 h, a drastic change in cell and nuclei shape is observed (Fig. 5, E). Cells are enlarged, and the cell volume at this stage is not reduced. The cells
become spindle-shape and more polarized with more pronounced cytoplasmic elongations. The size of the nuclei is increased; they have a smooth surface and have lost the typical irregular and incised form seen in the control. Most importantly, after 24 h, a dramatic accumulation of lipid vesicles in the cytoplasm is clearly visible (Fig. 5E). In almost each of the investigated tumor cells, lipid depositions can be seen near the nuclei. After 48 h, SAM-6-treated cells have reached the final stage of apoptosis (Fig. 5F). The most important structural changes include the disappearance of cell-cell contacts, cell shrinkage, high condensation of nuclei, and degradation of plasma and nuclear membrane (Fig. 5H).

Fig. 4. Scanning electron microscopy of SAM-6 antibody-induced apoptosis. Stomach carcinoma cell line 23132/87 was incubated with antibody SAM-6 and isotype control at a concentration of 10 μg/ml for the indicated periods of time. Samples were proceeded for scanning electron microscopy and analyzed by ZEISS DSM 962. A–C, isotype control antibody; D–F, SAM-6 antibody; bar indicates 20 μm. Magnification, ×3,800; bar indicates 20 μm. G–I, magnification of SAM-6 apoptotic effects. G, stress fibers, ×7,000; bar indicates 10 μm. H, nucleus swelling, ×20,000; bar indicates 2 μm. I, apoptotic bodies, ×40,000; bar indicates 2 μm.

Fig. 5. Transmission electron microscopy of SAM-6 antibody-induced apoptosis. Stomach carcinoma cell line 23132/87 was incubated with antibody SAM-6 and isotype control at a concentration of 10 μg/ml for the indicated periods of time. Samples were proceeded for transmission electron microscopy and analyzed by ZEISS 902. A–C, isotype control antibody; D–F, SAM-6 antibody; magnifications in A, B, D, and E, ×2750; in C and F, ×7500. G–I, magnification of SAM-6 apoptotic activity. G, arrows indicate intracellular lipid accumulation; magnification, ×30,000. H, arrows indicate nuclear membrane degradation, ×30,000. I, magnification of membrane vesicles (apoptotic bodies) formation and secretion from the surface of two apoptotic cells, ×17,500.
membranes. The higher magnifications show a cluster of lipid vesicles accumulated in tumor cells (Fig. 5G), nuclear membrane degradation (Fig. 5H), and formation of apoptotic bodies from the cell surface of two tumor cells (Fig. 5I).

**Sudan III Staining.** To examine the antibody-induced lipid accumulation, we performed a staining with Sudan III. This dye is specific for the detection of neutral lipids and fatty acids. Fig. 6 shows the data obtained after 48 h of incubation with antibody SAM-6, CD95/Fas antibody, or unrelated human control IgM on gastric cancer cells and pancreas carcinoma cells. The gastric carcinoma cell line 23132/87 clearly shows an antibody-induced accumulation of neutral lipids when treated with antibody SAM-6 (Fig. 6B). The same results were observed with the pancreas carcinoma cell line BXPC-3 treated with antibody SAM-6 (Fig. 6D). In contrast, the cells treated with unrelated human control IgM do not exhibit similar intracellular changes (Fig. 6, A and C). BXPC-3 cells treated with CD95/Fas antibody show morphologically a clear apoptotic effect but no accumulation of neutral lipids (Fig. 6F). These data indicate that the intracellular accumulation of lipids is a direct effect mediated by antibody SAM-6.

**Nile Red Staining.** Cellular lipids can also be visualized by staining with the fluorescence stain Nile red. Here, nonpolar or neutral lipids stain yellow-gold and polar lipids stain dark red when investigated at specific wavelengths (26, 27). Stomach cancer cells (23132/87) were incubated for 48 h with antibody SAM-6 and investigated for lipid accumulation. Fluorescence was measured at 488 nm for nonpolar or neutral lipids and at 543 nm for polar lipids. Fig. 7, A and D, shows yellow staining for nonpolar, neutral lipids; Fig. 7, B and E, red staining for polar lipids; and Fig. 7, C and F, an overlay of both. As expected, an intense yellow fluorescence stain for neutral lipids in the SAM-6-treated cells can be seen after 48 h (Fig. 7D). An increase is visible for SAM-6-treated cells stained for polar lipids (Fig. 7E), compared with the control (Fig. 7B), indicating a higher amount of membrane proteins. Because the antibody SAM-6 induces apoptosis, the higher amount of polar lipids is most likely the result of more membrane vesicle formation, namely apoptotic bodies. In the overlay, seen in Fig. 7, C and F, polar lipids are seen in red and neutral/nonpolar lipids are in yellow; and some are in orange, as expected. Although the red fluorescence of Nile red is very intense and there might be a possible red spill-over into the yellow-gold fluorescence measurement, a clear distinction can be made between the neutral/nonpolar and polar lipid staining. Taken together these results show, in addition to the Sudan III stain, that the SAM-6 antibody induces neutral/nonpolar lipid accumulation in cancer cells.

**Toxicity in Vivo.** To examine whether antibody SAM-6 shows any toxic effects in vivo, mice received i.p. injections of either purified SAM-6 antibody or unrelated human control IgM. After 2 and 14 days, mice were sacrificed, organs were inspected for toxic changes, and blood samples were measured for liver enzymes. Furthermore,
paraffin sections of embedded organs were stained with Sudan III to examine possible lipid accumulations. In general, antibody-treated mice showed no toxic side effects, and the antibody was well tolerated. The organs of SAM-6-treated mice showed no alterations in comparison with the organs of control mice. No toxic changes could be observed in both groups. In addition the organs of both groups did not show any lipid accumulation at all. The liver enzymes were not altered in mice treated with antibody SAM-6 compared with control mice.

DISCUSSION

A balanced lipid homeostasis is crucial for not only healthy but also for malignant cells. The natural, germ-line coded IgM antibody SAM-6 binds to a $M_r 140,000$ membrane receptor on human epithelial cancer cells. The antibody increases the lipid content and induces apoptosis of tumor cells, but not of normal nontransformed human epithelial cells, indicating tumor specificity of the receptor. Lipoptosis, antibody-mediated intracellular lipid accumulation followed by apoptosis, might be a new approach to fight cancer.

A balanced lipid metabolism is crucial for all cells, and extensive lipid storage can result in apoptotic cell death. This was shown in several animal studies and was also described for some inherited and acquired human diseases (2, 3). Some metabolic mechanisms of lipo-apoptosis have been described, and some causes are discussed, but reagents, which directly induce these syndromes, have thus far not been identified. The human antibody SAM-6, which was isolated from a gastric cancer patient, specifically binds to epithelial cancer cells. Binding of SAM-6 to malignant cells induces a specific form of apoptosis, named lipoptosis. This deadly process is characterized by an extreme intracellular accumulation of lipid vesicles. This lipoptotic effect is specific for the antibody SAM-6, because other apoptotic human IgMs (10, 11) or the murine antibody CD95/Fas do not show this unique feature.

Apoptosis is not only the fastest, but also the cleanest way to remove unwanted cells. This form of cellular recycling without strong inflammatory processes, as in the case of complement lyses, seems to be the best way not only of cellular elimination in differentiation processes, but also for immunosurveillance mechanisms (28). It is likely that nature makes use of different receptors and mechanisms of inducing apoptosis, depending on the IgM antibody and the type of receptor. The SAM-6 lipoptotic pathway, the characterization of which is under way as well as the determination of the neutral/nonpolar lipids, is a very good example of nature’s variety of skills in eliminating malignant cells. There might be a good chance that the characterization of additional natural antibodies and their receptors will give even more different examples of the “apoptotic fantasy” in immunosurveillance processes.

The antibody SAM-6 is a natural IgM and part of the immunosurveillance mechanisms against malignant epithelial cells (10). Malignancy is like a chronic disease, and the immune system is permanently involved in eliminating transformed cells. During its lifetime, each multicellular organism is permanently exposed to transformed cells, which arise spontaneously or by inducing factors. Even if not every transformed cell has the ability and potency for malignant behavior, the important question is not how malignant cells arise, but instead, why malignancy occurs so infrequently (29). An early recognition and a rapid elimination of transformed cells by immune mechanisms is essential for keeping an organism free of tumor cells, and mechanisms of the innate immunity, especially IgM antibodies, were shown to play a major role in these recognition and elimination processes (10).

The antibody SAM-6 binds to a membrane protein with a molecular weight of $M_r 140,000$, the expression of which is tumor-specific, because nontransformed human epithelial and fibroblastic cells do not bind the antibody. Malignant epithelial cells do not seem to create “new” tumor-specific structures on the cells surface, because all of the tumor-specific human monoclonal IgM antibodies described thus far recognize antigens, which are posttranscriptionally modified (13–15). The epitopes are tumor-specific N-linked carbohydrate structures on “normal” cell surface molecules, like decay acceleration factor/CD55 or cysteine-rich fibroblast growth factor receptor 1 (10, 14, 15). An abnormal glycolipid and glycoprotein synthesis by tumor cells very often results in expression of these modified structures on the surface (30, 31). For the detection of malignant cells, innate immune receptors seem to recognize these tumor-specific carbohydrate structures, because they are conservative and expressed independently from mutational events (32). This recognition system guarantees that the immune response targets the right cells.
response need not follow all mutational changes but can focus on the detection of structures, which are most likely involved in primary cell stability and cell preservation mechanisms and not in recognition processes. The biochemical analysis of the SAM-6 receptor and epipode is under way and needs to be awaited.

The antibody SAM-6 is coded by the germ-line genes IgHV3-30.3*01 and IgLV3-1*01. A series of other tumor-specific natural IgM antibodies described thus far belong to the same or similar germ-line gene families (10). The innate immunity is, in contrast to the adapted immunity, not trainable. The immune-competent cells fulfill their task by using specific sets of germ-line coded receptors, which belong to distinct protein families, coded by only a limited number of germ-line genes (32, 33). The acquired genetic variability of the innate immunoglobulin receptors is achieved by combinatorial association of germ-line immunoglobulin genes. Additional deletions, additions, and mistakes in recombination events guarantee a genetic and receptor variability, which is sufficient to cover a broad spectrum of antigens on pathogenic organisms and gives a sufficient protection without additional mutational adaptation (34–40). Interestingly, the reactivity pattern of all these IgMs correlates reciprocally with their mutational status, which means that with increasing mutation frequency, the grade of reactivity to other tumors decreases (10).

In summary, it is obvious that the innate immunity and natural IgMs play an important role in immunosurveillance mechanisms against epithelial tumors in humans and that human natural IgM antibodies are ideal tools to fight cancer (10). After 15 years of experience with the human antibody technology and human IgMs, the majority of the technical problems concerning low affinity and cross-reactivity of human IgM antibodies are nearly solved (41). Several interesting human natural IgM antibodies have already been established that are useful for diagnostic purposes and have been successfully used in clinical trials (13, 21, 22). We describe in this paper a new natural IgM antibody, SAM-6, that induces a deadly intracellular accumulation of lipids in malignant cells. This new mechanism of tumor-specific apoptosis, named lipoptosis, might be a new approach for therapeutic purposes.

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