Mechanism of apoptosis induced by S100A8/A9 in colon cancer cell lines: the role of ROS and the effect of metal ions

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Abstract: The protein complex S100A8/A9, abundant in the cytosol of neutrophils, is secreted from the cells upon cellular activation and induces apoptosis in tumor cell lines and normal fibroblasts in a zinc-reversible manner. In the present study, we present evidence that the S100A8/A9 also exerts its apoptotic effect by a zinc-independent mechanism. Treatment of the colon carcinoma cells with different concentrations of human S100A8/A9 or the metal ion chelator diethylenetriaminepentacetic acid (DTPA) resulted in a significant increase of cell death. Annexin V/phosphatidylinositol and Hoechst 33258 staining revealed that cell death was mainly of the apoptotic type. A significant increase in the activity of caspase-3 and -9 was observed in both cell lines after treatment. Caspase-8 activation was negligible in both cell lines. The cytotoxicity/apoptotic effect of human S100A8/A9 and DTPA was inhibited significantly ($P<0.05$) by Zn^{+2} and Cu^{+2}, more effectively than by Ca^{2+} and Mg^{2+}. The antioxidant N-acetyl-L-cysteine inhibited the cytotoxicity/apoptotic effect of S100A8/A9 and DTPA. However, as a result of the different time-courses of both agents and that the S100A8/A9-induced apoptosis was not completely reversed, we conclude that S100A8/A9 exerts its apoptotic effect on two colon carcinoma cell lines through a dual mechanism: one via zinc exclusion from the target cells and the other through a yet-undefined mechanism, probably relaying on the cell-surface receptor(s). *J. Leukoc. Biol.* 76: 169–175; 2004.

Key Words: *caspase activation · polymorphonuclear neutrophils · cytotoxic peptides · calcium-binding protein · zinc-binding protein*

INTRODUCTION

Polymorphonuclear neutrophils (PMNs), a vital component of the innate-immune response, perform several host-defense functions, such as phagocytosis of invading microorganisms and cell debris, release of a number of arachidonic acid-derived eicosanoids, generation of reactive oxygen species (ROS), and release of proteolytic enzymes as well as bactericidal and cytotoxic peptides. Activated phagocytes have also been shown to specifically secrete the protein complex S100A8/A9 in the extracellular environment [1], and extracellular S100A8/A9 exerts antimicrobial activity [2–5] as well as an apoptotic/cytotoxic effect against various tumor cells [6] and normal cell types, including myeloid cells [7], mitogen-activated lymphocytes [8, 9], and normal fibroblasts [10].

The antimicrobial activity appears to depend on the ability of S100A8/A9 to sequester zinc efficiently, enough so that free concentrations of Zn^{2+} fall below the low levels needed by most microorganisms [11, 12]. It has been assumed that the binding of bivalent metal ions by S100A8/A9 is also involved in the cytotoxity/apoptotic effect [10]; however, the underlying mechanism of its apoptotic/cytotoxic effect is still unknown.

The protein complex S100A8/A9 is formed by the two low molecular weight calcium-binding proteins S100A8 and S100A9 belonging to the S100 protein family (for review, see refs. [13, 14]). The expression of the two S100 proteins is restricted to a specific stage of myeloid differentiation, probably driven by a recently characterized regulatory element [15]. In addition to the binding to Ca^{2+}, S100A8/A9 has been shown to bind other bivalent cations, such as Zn^{2+} and Cu^{2+} [4, 16–19]. The binding motif for these bivalent cations is still in debate [17, 18, 20]. It is interesting that one putative zinc-binding site has been associated with the specific binding of polyunsaturated fatty acids, another feature of S100A8/A9 [21–23]. Moreover, it has been reported that antimicrobial activity and cell death (apoptosis)-inducing activity of S100A8/A9 are inhibited in the presence of zinc [10, 24].

With respect to the above-mentioned biological activity of S100A8/A9, this study was performed to investigate the underlying mechanism of apoptosis by S100A8/A9 in the colon cancer using HT29/219 and SW742 colon carcinoma cell

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lines. Furthermore, we investigated the effect of bivalent ions on the S100A8/A9-induced effect to benefit from its therapeutic effect in the management of colon cancer.

MATERIALS AND METHODS

Chemicals, culture media, and related compounds were purchased from Sigma Chemical Co. (St. Louis, MO). All additives have been tested for endotoxin contaminations. Cell-culture plasticware was obtained from Nunc Co. (Roskilde, Denmark); caspase-3 colorimetric assay kit (Cat. No. 101K4019) and caspase-8 colorimetric assay kit (Cat. No. 80K4104), from Sigma (Germany); and caspase-9 colorimetric assay kit (Cat. No. BF10100) and annexin V–fluorescein isothiocyanate apoptosis detection kit (Cat. No. TA4638), from R&D Systems (Minneaspolis, MN).

Purification of S100A8 and S100A9 from human neutrophils

Human neutrophils were prepared from leukocyte-rich blood fractions ("buffy coat") according to Müller et al. [25]. S100A8/A9 was purified as described by van den Bos et al. [26] with minor modifications. Prior to use, the proteins were rechromatographed by anion exchange chromatography using UnoQ column (Bio-Rad, Munich, Germany).

Cell culture

HT29/219 [National Cell Bank of Iran (NCBI) C154] and SW742 (NCBI C146) colon carcinoma cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. They were incubated at 37°C in a humidified CO_2 incubator with 5% CO_2 and 95% air. Cultures were examined regularly.

Cytotoxicity assay

To evaluate the cytotoxicity effect of S100A8/A9 and diethylenetriaminepentacetic acid (DTPA) on these cell lines, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay was applied [27]. Briefly, asynchronously growing cells (1.5×10^4 cells/ml) were transferred into 96-well culture plates containing 200 μl medium and incubated for 24 h. Various concentrations of S100A8/A9 or DTPA were added and incubated for different time intervals, as indicated, followed by MTT assay. The percentage of cell viability was calculated using the equation: [mean optical density (OD) of treated cells/mean OD of control cells] × 100.

Analysis of nuclear morphology

Cells were plated in eight-well chamber slides and allowed to adhere. S100A8/A9 and DTPA-treated cells were fixed with methanol-acetic acid 3:1 (v/v) for 10 min, after which staining was performed with Hoechst 33258 (200 μg/ml). Slides were then washed with phosphate-buffered saline (PBS; pH 7.4) and examined by an epifluorescence microscope (Micros, Austria). Apoptotic cells were defined on the basis of nuclear morphology changes such as chromatin condensation and fragmentation

Caspase-3, -8, and -9 activation assays

A caspase-3 [using Asp-Glu-Val-Asp (DEVD)–pNA as substrate], caspase-8 [using Ac-Ile-Glu-Thr-Asp (IETD)–pNA as substrate], and caspase-9 [using Leu-Glu-His-Asp (LEHD)–pNA as substrate] colorimetric assay kits were used to investigate the activation of these caspases in the treated HT29/219 and SW742 cells. Briefly, to estimate caspase-3 and -8 activity, cells were lysed by incubation with cell lysis buffer on ice for 15 min and then centrifuged at 20,000 *g* for 10 min (at 4°C). For caspase-9 activation assay, cells were lysed by incubation with cell lysis buffer on ice for 10 min and then centrifuged at 10,000 *g* for 1 min (at 4°C). Enzymatic reactions were performed in a 96-well flat-bottom microplate. To each reaction samples, 5, 25, and 50 μl cell lysate (100–200 μg total protein) was added for caspase-3, -8, and -9, respectively. Additional controls, one free from cell lysate and the other lacking substrate as well as caspase-3- and -8-positive controls, have been included. Protein content was estimated by the Bradford method [28]. The activities were expressed as nmole/min/mg protein.

Quantification of Zinquin fluorescence by fluorimetry

Zinquin was used to estimate the intracellular zinc concentrations as described prevoiusly [29, 30]. Briefly, after treatment with S100A8/A9 or DTPA for 24 h, 10^6 cells were incubated in PBS containing 1 mg/ml ovalbumin and 25 μM Zinquin for 30 min (cells for each test). Zinquin was diluted fresh in PBS (pH 7.4) and used immediately, preferably added directly to the cells. After 30 min at room temperature, the cells were transferred into fluorimetry grade cuvettes, and the fluorescence was measured at excitation/emission wavelengths of 365/490 nm in a Shimadzu RF 5000 spectrofluorimeter.

Effect of various divalent metal ions on S100A8/A9 and DTPA cytotoxicity activity

To evaluate the effect of different divalent metal ions (calcium, magnesium, copper, zinc), the cell lines were treated with DTPA (100 μM) or S100A8/A9 (150 μg/ml) for 48 h in the presence of increasing concentrations of metal ions as indicated.

Effect of N-acetyl-L-cysteine (NAC) on S100A8/A9 and DTPA cytotoxicity effect

To study the involvement of ROS in the induction of apoptosis by S100A8/A9, the cell lines were pretreated with increasing concentrations of NAC for 24 h. The cell lines were then treated with S100A8/A9 (150 μg/ml) or DTPA (100 μM) for 48 h.

Statistical analysis

The results were expressed as the mean ± SD, and statistical differences were evaluated by one-way ANOVA. $P < 0.05$ was considered significant.

RESULTS

Cytotoxicity assay

To determine the cytotoxicity/apoptotic activity of S100A8/A9, viability tests were applied using MTT assay at different concentrations and time intervals as indicated. For control, we used in analogous experiments the membrane-impermeable zinc chelator, DTPA. As shown in **Figure 1**, treatment of colon carcinoma cell lines with S100A8/A9 or DTPA resulted in significant cell death. However, both cell lines showed a remarkable difference in their sensitivity toward both agents regarding time-course and effective doses.

The treatment of HT29/219 cells with human S100A8/A9 resulted in significantly reduced cell viability at concentrations higher than 120 μg/ml within 12 h. At 30 h, a value of 50% viability was determined at concentrations higher than 120 μg/ml (corresponding to 5 μM S100A8/A9, Fig. 1A). HT29/219 cells treated with DTPA showed no significant cell death after 12 and 24 h. At 36 h, a value of 50% viability was determined at concentrations higher than 40 μM (Fig. 1B).

The S100A8/A9-treated SW742 cells showed a significant cell death at all time intervals (Fig. 1A, right). At 12 h, a value of 50% viability was determined at concentrations higher than 120 μg/ml S100A8/A9 (Fig. 1A). In the SW742 cell line, DTPA induced a significant cell death ($P<0.05$) at concentrations higher than 60 μM at 36 h; however, a value of 50% cell viability was not determined at all time intervals in the concentration range of 20–100 μM DTPA.

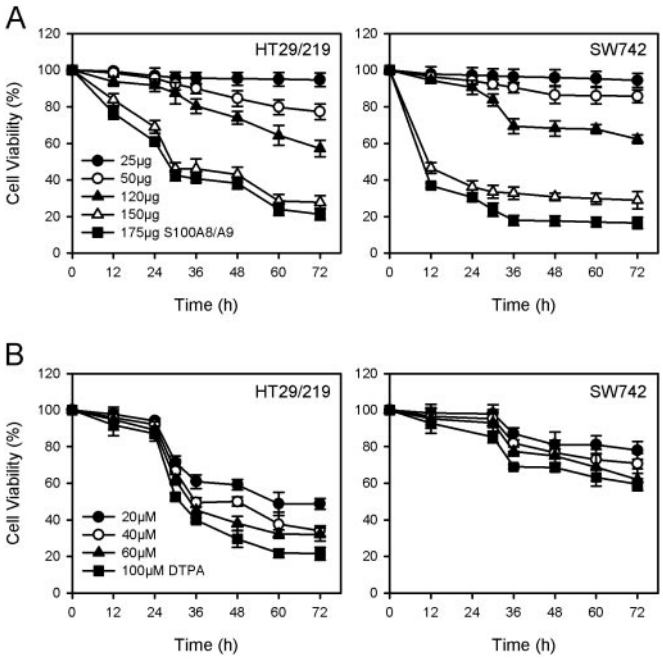

Fig. 1. Effect of S100A8/A9 (A) and DTPA (B) on the growth of HT29/219 and SW742 cell lines. The cells were treated with different concentrations of S100A8/A9 and DTPA for 12–72 h, and the viability was assessed by MTT assay. Results are expressed as percentage of corresponding control and represent the mean ± SD of four repeats.

To verify whether the observed proapoptotic effect of S100A8/A9 was specific, we also determined the cytotoxicity/apoptotic activity of the individual S100 proteins S100A8 and S100A9. At 24 h, a value of 50% viability was determined at concentrations higher than 400 μg/ml S100A8 (corresponding to 36 μM) and 300 μg/ml S100A9 (corresponding to 21 μM) for both cell lines, respectively (data not shown). Both individual S100 proteins were shown to bind Zn^{2+} [31, 32]. These results indicate that S100A8/A9 exerts cytotoxicity/apoptotic activity against both colon carcinoma cell lines. Moreover, as a result of the different time-courses of S100A8/A9 and DTPA, we assumed that the underlying mechanism was not a simple sequesteration of zinc or at least that could not be the sole means of action.

Detection of apoptosis by Hoechst 33258 staining

To get further insights in the underlying cell death mechanisms, we examined the morphology of dying cells upon treatment with S100A8/A9 and DTPA. The cell morphology was first examined by light-phase contract microscopy. As obvious from **Figure 2**, the changes in the morphology of both cell lines treated by S100A8/A9 were different from those observed with DTPA.

To confirm the apoptotic cell death, cell nuclei were stained with Hoechst 33258. As shown in **Figure 3** (right), the S100A8/A9 protein complex caused typical apoptotic changes in the nuclear morphology, with pronounced condensation of cell nuclei and nuclear fragmentation. The apoptotic changes are more pronounced in HT29/219 than in SW742. Thus, the

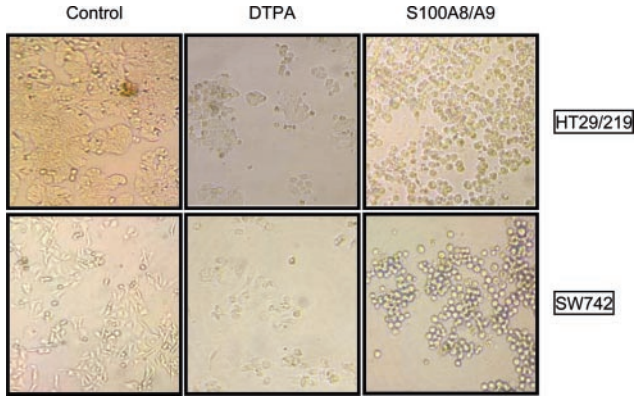

Fig. 2. Morphology of HT29/219 [upper: left, Control; middle, DTPA (80 μM); right, S100A8/A9 (150 μg/ml)] and SW742 [lower: left, Control; middle: DTPA (80 μM); right, S100A8/A9 (150 μg/ml)] cell lines after treatment with DTPA or S100A8/A9 for 36 h by invert microscopy.

morphology picture corresponds well to the data obtained previously by the MTT assay.

The detection of caspase-3, -8, and -9 activation

To explore the mechanisms underlying human S100A8/A9 and DTPA-induced apoptosis, the activation of caspase-3, -8, and caspase-9 was examined using semispecific DEVDase-, IETDase-, and LEHDase-enzymatic assays. The results demonstrated that the activity of caspase-3 and -9 was significantly ($P<0.05$) increased in both cell lines treated with human S100A8/A9 or DTPA (**Fig. 4, A** and **C**). The S100A8/A9 was a much better apoptotic inducer than DTPA. It caused about twofold higher activation of caspase-3 and caspase-9 as compared with DTPA. There was comparatively only a slight increase in the activity of caspase-8 in both cell lines treated with S100A8/A9 or DTPA at a concentration above 120 μg/ml for S100A8/A9 (Fig. 4B, left) and above 100 μM for DTPA (Fig. 4B, right), respectively.

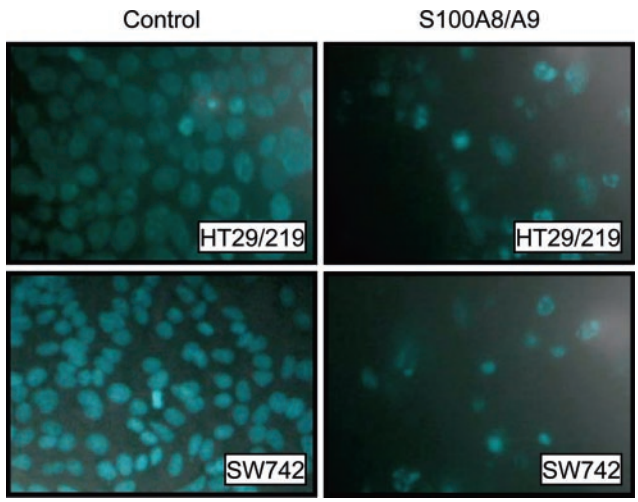

Fig. 3. Detection of typical features for apoptosis nuclear condensation upon stimulation of HT29/219 cells with S100A8/A9 (calprotectin) by Hoechst 33258 staining.

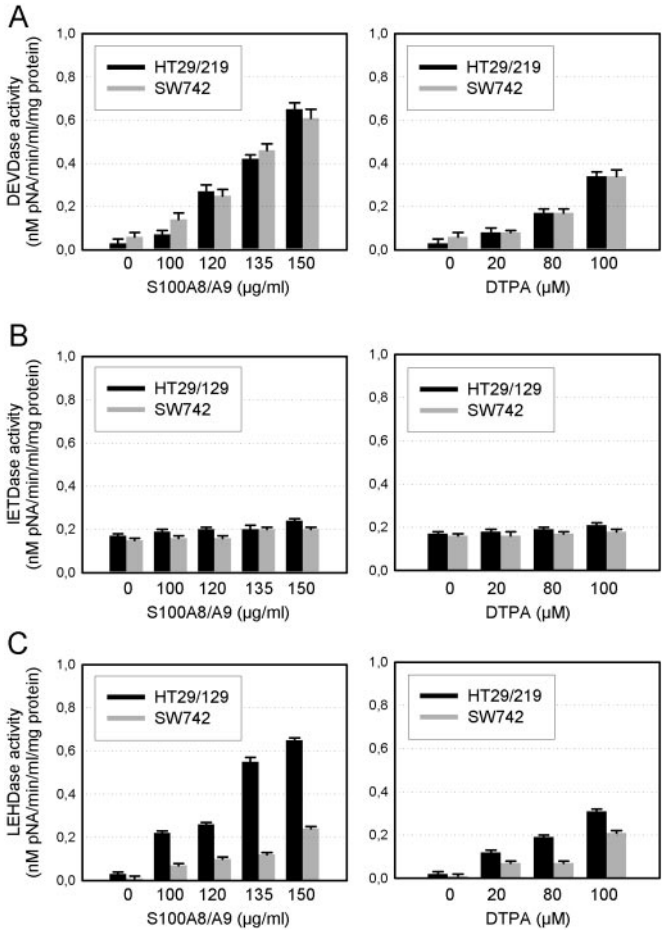

Fig. 4. Enzymatic measurement of activity of caspase family of proteases. Activity of caspase-3 (DEVDase activity; A), caspase-8 (IETDase activity; B), and caspase-9 (LEHDase activity; C) in HT29/219 and SW742 cells following treatment with human S100A8/A9 and DTPA for 36 h was quantified by an enzymatic assay (see Materials and Methods for details). Results are expressed as activity of the enzyme and represent the mean ± SD of four repeats.

Detection of intracellular zinc by Zinquin

Depletion of intracellular zinc decreases the intracellular pool of this ion, thus resulting in activation of caspase-3 [33, 34]. To analyze the mechanisms underlying human S100A8/A9 and DTPA-induced apoptosis, the intracellular zinc concentrations were detected using the Zinquin fluorescence assay. The results confirmed that the intracellular zinc concentration was decreased in both cell lines treated with human S100A8/A9 or DTPA (**Fig. 5**). However, DTPA was less effective in depletion of intracellular zinc in the SW742 cell line in accordance with the reduced apoptosis-inducing activity of DTPA in these cells.

The effect of divalent metal ions (calcium, magnesium, zinc, and copper) on the S100A8/A9- and DTPA-induced apoptosis

As it has been proposed previously that the divalent cation-chelating activity of S100A8/A9 is the mechanism responsible for its toxicity, we investigated the effect of divalent metal ions on the cytotoxicity induced by S100A8/A9 or by DTPA (**Fig. 6**). HT29/219 and SW742 cell lines were treated by S100A8/A9 (150 μg/ml) or DTPA (100 μM) in the presence

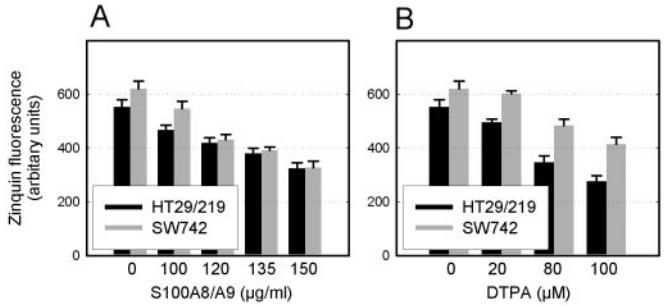

Fig. 5. The effect of S100A8/A9 (A) and DTPA (B) on intracellular zinc concentration by Zinquin in the HT29/219 and SW742 colon cell lines.

and absence of different concentrations of calcium, magnesium, zinc, and copper. A significant, reduced apoptotic effect of S100A8/A9 and DTPA was observed after the addition of zinc and copper, whereas calcium and magnesium had no modulatory activity. It is worthwhile mentioning that the addition of zinc or copper did not fully reverse the apoptotic effect of S100A8/A9 and DTPA, thus bivalent cation-chelating-independent mechanism(s) are likely also involved in the observed cell death.

The effect of NAC on the S100A8/A9- or DTPA-induced apoptosis

The activation of caspase-9 is characteristic for the classical mitochondrial, cytochrome c-dependent pathway. The bivalent cation supplementation experiments showed that a chelating-independent mechanism was also likely responsible for S100A8/A9 toxicity. In addition, death induced by some stim-

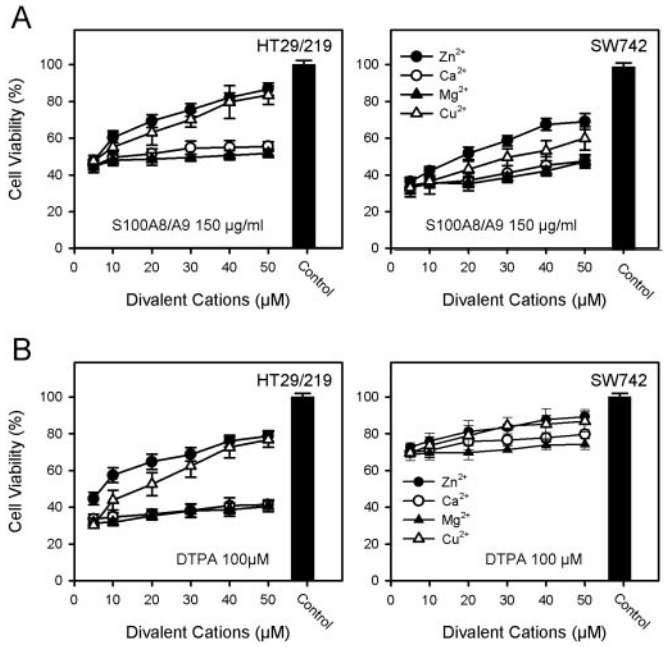

Fig. 6. The effect of divalent metal ions on the cytotoxicity of human S100A8/A9 (A) and DTPA (B) in HT29/219 and SW742 colon cell lines. Cells were treated with the indicated stimuli for 48 h. The cell death was detected by MTT assay. Results are expressed as activity of the enzyme and represent the mean ± SD of four repeats.

uli, including tumor necrosis factor-α, significantly relies on ROS production by mitochondria [35, 36]. To get further insights into S100A8/A9 toxicity pathways, we examined the effects of NAC, a broadly used clinical antioxidant. As shown in **Figure 7**, NAC potently and in a dose-dependent manner protects from S100A8/A9 and DTPA toxicity. Although NAC showed a typical linear-dose-dependent mean of action upon DTPA treatment, and up to 10 mM concentration is required to fully counteract the stimulus, 5 mM NAC was sufficient for complete reversal of the S100A8/A9 toxicity. These results further support our hypothesis that the mean of action of S100A8/A9 differs significantly from the divalent cation chelator DTPA.

DISCUSSION

There are several reports indicating that rat S100A8/A9 or human recombinant S100A8/A9 induces apoptosis in various human or mouse tumor cell lines [6–10]. It has been assumed that the apoptotic activity was a result of the sequesteration of zinc. However, in the present study, we present evidence that S100A8/A9 induces apoptosis by a second mechanism that is independent from the sequestration of zinc ions.

S100A8/A9 was able to decrease the MTT-reducing activity of both colon carcinoma cell lines, although HT29/219 cells were significantly more sensitive than SW742 cells. The apotosis-inducing activity of S100A8/A9 was characterized by Hoechst 33258 staining. The effective concentration of S100A8/A9 was in a range comparable with those of other reports. In contrast to Huttunen et al. [37], we did not observe any promoting effect upon cell survival at nM concentrations of S100A8/A9 (data not shown).

However, the apoptotic activity of S100A8/A9 showed remarkable divergences to the DTPA-induced apoptosis. DTPA, the membrane-impermeable metal ion chelator, induces apoptosis through the depletion of extracellular zinc ion [24]. S100A8/A9 also binds zinc with high affinity, and it has been reported that the antimicrobial activity and the cell death (apoptosis)-inducing activity of S100A8/A9 are inhibited by the presence of zinc [10, 24, 38]. However, based on the observations that Zn^{2+} and Cu^{2+} did not completely reverse the apoptotic effect of S100A8/A9; the changes in the morphology of both cell lines treated by S100A8/A9 were different from those observed with DTPA; and the time-course of S100A8/A9-induced apoptosis differed from that induced by DTPA, we conclude that S100A8/A9 induces apoptosis by a mechanism that is not simple as a result of zinc sequestration. In addition, although the individual S100 proteins also bind zinc [31, 32], S100A8/A9 was a much more potent inducer of apoptosis. These observations expand our knowledge about the mechanism of apoptotic action of S100A8/A9 complexes, showing that in addition to the Zn^{2+} activity described in earlier studies [24], an additional Zn^{2+}-independent mechanism exists.

The S100A8/A9-induced apoptotic activity was induced through the classical mitochondrial, cytochrome c-dependent (extrinsic) pathway, as verified by the activation of caspase-9 (and caspase-3) but not by activation of caspase-8. The finding that caspase-8 activity was only slightly increased after S100A8/A9 treatment clearly indicates that the caspase-8/death receptor pathway was not involved in the S100A8/A9-induced apoptosis. It has been shown recently that the caspase-3 zymogen (pro-caspase-3) is stabilized in the presence of zinc ions, directly through binding to Zn^{2+} [33, 34] or indirectly through the effect of Zn^{2+} on redox-controlled processes [39]. Thus, extracellular chelation of zinc by S100A8/A9 or DTPA might decrease the intracellular pool of this ion, thus resulting in the activation of caspase-3. However, in our study, DTPA induced caspase-3 in both cell lines at similar levels, although DTPA showed significant, different effects on the intracellular zinc level, indicative for a zinc-independent mechanism. Independently, intracellular Zn^{2+} depletion causes significant cellular stress by itself, as these bivalent cations are critical for function of several transcription factors and enzymes. Cellular stress is known to activate the mitochondrial/apoptosome-dependent (intrinsic) pathway (for review, see refs. [40, 41]).

ROS, which are the byproducts of normal cellular oxidative processes, have been suggested as regulating the process involved in the initiation of apoptotic signaling. In our study, we were able to show that the pretreatment of the cells with the antioxidant NAC prevented apoptosis induced by S100A8/A9. Therefore, a facilitation of a pro-oxidant state likely contributes to the molecular mechanism by which S100A8/A9 exerts its apoptotic effect. However, although S100A8/A9 showed a

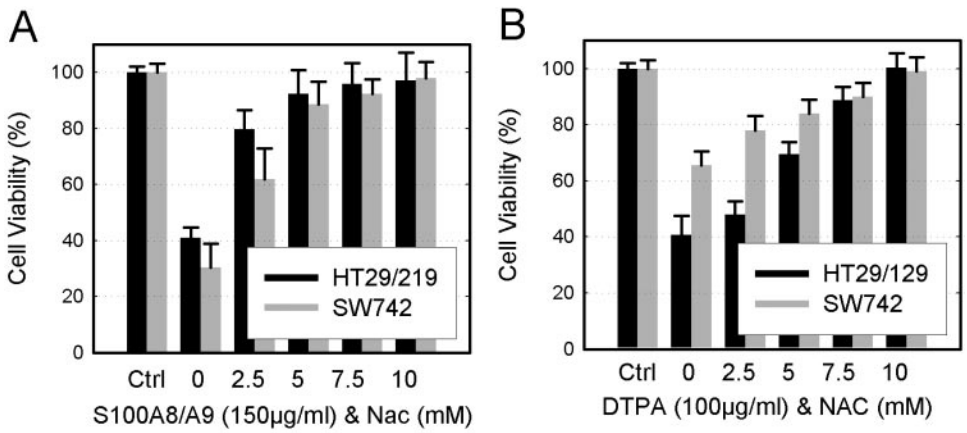

Fig. 7. The effect of NAC on the cytotoxic effect of human S100A8/A9 (A) and DTPA (B) in HT29/219 and SW742 colon cell lines. The cells were treated with the indicated stimuli for 48 h. The cell death was detected by MTT assay. Results are expressed as activity of the enzyme and represent the mean ± SD of four repeats.

higher apoptotic potency and a higher stimulatory effect on caspase-3 and caspase-9 activation, lower concentrations of NAC were able to reverse the S100A8/A9-induced apoptosis. In the control experiments, there was a linear decrease of DTPA-induced apoptosis with increasing concentrations of NAC. Therefore, we conclude that S100A8/A9 induced apoptosis through a dual mechanism: One might present zinc exclusion from the target cells, and the other might be through binding to the cell surface of the target cells, possibly in a ligand-receptor manner.

Several binding sites for S100A8/A9 have been reported on various human leukemia [42] and endothelial cells [43–47]; however, the cell-surface receptor of S100A8/A9 is still in debate. The binding site(s) on the colon carcinoma cell lines by which S100A8/A9 induces its apoptotic effect are the issue of our current research. However, our demonstration showed that S100A8/A9 exerts apoptotic activity in target cells, possibly in a ligand-receptor manner. Together with a recent report demonstrating the accumulation of S100A8- and S100A9-positive cells, macrophages, and PMNs, along the invasive margin of carcinoma [48], our study points to the possible participation of S100A8/A9 in carcinoma regression.

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