Fermented wheat germ extract induces apoptosis and downregulation of major histocompatibility complex class I proteins in tumor T and B cell lines

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Abstract. The fermented wheat germ extract (code name: MSC, trade name: Avemar), with standardized benzoquinone content has been shown to inhibit tumor propagation and metastases formation *in vivo*. The aim of this study was to understand the molecular and cellular mechanisms of the anti-tumor effect of MSC. Therefore, we have designed *in vitro* model experiments using T and B tumor lymphocytic cell lines. Tyrosine phosphorylation of intracellular proteins and elevation of the intracellular Ca^{2+} concentration were examined using immunoblotting with anti-phosphotyrosine antibody and cytofluorimetry by means of Ca^{2+} sensitive fluorescence dyes, Fluo-3AM and FuraRed-AM, respectively. Apoptosis was measured with cytofluorimetry by staining the DNA with propidium iodide and detecting the 'sub-G_1' cell population. The level of the cell surface MHC class I molecules was analysed with indirect immunofluorescence on cytofluorimeter using a monoclonal antibody to the non-polymorphic region of the human MHC class I. MSC stimulated tyrosine phosphorylation of intracellular proteins and the influx of extracellular Ca^{2+} resulted in elevation of intracellular Ca^{2+} concentration. Prominent apoptosis of 20-40% was detected upon 24 h of MSC treatment of the cell lines. As a result of the MSC treatment, the amount of the cell surface MHC class I proteins was downregulated by 70-85% compared to the non-stimulated control. MSC did not induce a similar degree of apoptosis in healthy peripheral blood mononuclear cells. Inhibition of the cellular tyrosine phosphatase activity or Ca^{2+} influx resulted in the opposite effect increasing or diminishing the Avemar induced apoptosis as well as the MHC class I downregulation, respectively. A benzoquinone component (2,6-dimethoxi-p-benzoquinone) in MSC induced similar apoptosis and downregulation of the MHC class I molecules in the tumor T and B cell lines to that of MSC. These results suggest that MSC acts on lymphoid tumor cells by reducing MHC class I expression and selectively promoting apoptosis of tumor cells on a tyrosine phosphorylation and Ca^{2+} influx dependent way. One of the components in MSC, 2,6-dimethoxi-p-benzoquinone was shown to be an important factor in MSC mediated cell response.

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Abbreviations: MHC, major histocompatibility complex; NK, natural killer; DMBQ, 2,6-dimethoxi-p-benzoquinone; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; TCR, T cell receptor; BCR, B cell receptor; mAb, monoclonal antibody; PMSF, phenylmethyl-sulfonylfluoride; pNPP, para-nitrophenyl-phosphate; PHA, phytohemagglutinine

Key words: fermented wheat germ extract, Avemar, MSC, benzoquinone, tyrosine phosphorylation, intracellular Ca^{2+}, CD45 tyrosine phosphatase, MHC class I downregulation, apoptosis

Introduction

Tumor cells acquire the ability of endless growth by escaping the control of proliferation and homeostasis of the normal cell. Among other mechanisms, there are two strategies used by tumor cells to survive: one is the newly acquired resistance toward apoptotic signals (1-3) and the other is an escape from the immunological surveillance (4,5). Apoptosis maintains the normal development and homeostasis of multi-cellular organisms. The process is characterized by typical morphological and biochemical alterations of the cell, including shrinking of the apoptotic cells, random DNA fragmentation,

expression of apoptotic receptors of the tumor necrosis factor receptor superfamily and activation of a specific protease cascade (6). Additional features of the cells undergoing apoptosis are the appearance of a sub-G_1 cell population emerging from cells with decreased DNA content and exposure of phosphatydilserine on outer membrane (6).

Tumor cells are capable of avoiding the adaptive immune response of cytotoxic T lymphocytes by expressing only self-antigens instead of tumor specific antigens in the context of MHC class I molecules (4) or by downregulation the MHC class I from the cell surface. Although the downregulation of MHC class I prevents the killing by cytotoxic T cells, the tumor cells become susceptible to natural killer (NK) cell activity (5).

The extract of wheat germ fermented with yeast, MSC, has been recently developed with standardized benzoquinone content. It has been previously shown that MSC exerts a potent anti-metastatic activity in animal tumor models (7). Moreover, interim results of a still running, phase II clinical trial with MSC indicate that it is an encouraging candidate of a supportive therapy after surgery and/or adjuvant chemotherapy in colorectal human malignancies (8). MSC treatment of thymectomized mice resulted in shortened rejection time of skin graft indicating that the graft, became sensitized to the immune response (9). MSC also decreased nucleic acid ribose synthesis through the non-oxidative steps of the pentose cycle but increased a direct glucose oxidation through the oxidative steps thus limiting cell proliferation and protecting human cells from oxidative stress (10). MSC is a mixture of ingredients with potential biological activity. One family of the active compounds may be benzoquinones, 2,6-dimethoxi-p-benzoquinone (DMBQ) and 2-methoxi-p-benzoquinone that are released by glycosidases present in the fermenting yeast. Although benzoquinones exert a strong effect on tumor cell proliferation (11), we have previously shown that benzoquinone may not be the only active compound in MSC (9).

To gain insight into the cellular and molecular mechanisms by which MSC exerts its anti-tumor effect, the early and late cell response have been studied upon *in vitro* treatment of lymphoid tumor cells with MSC. Early bio-chemical events such as induction of tyrosine phosphorylation, modulation of tyrosine phosphatase activity and elevation of intracellular Ca^{2+} concentration have been investigated. Furthermore, the biological response of the lymphoid tumor cells including expression of the cell surface MHC class I proteins and induction of programmed cell death upon MSC treatment have been analysed.

Materials and methods

Cells and reagents. The wild-type and two mutant phenotypes of *Jurkat* leukemic T cells, the $p56^{lck}$ and CD45 deficient variants, *JCam* and *J45.01*, respectively, the Burkitt lymphoma B cell lines, *Bl41* and *Raji* and the myelo-monocytic cell line, *U937* were cultured in RPMI-1640 (Gibco, Rockville, MD, USA) medium containing 5% fetal calf serum (FCS) (Protein GMK) at 37˚C under 5% CO_2. Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by density gradient centrifugation over Ficoll-Hypaque.

2,6-dimethoxi-p-benzoquinone (DMBQ) was chemically synthetized in our laboratory. Reagents were purchased from Sigma (St. Louis, MO, USA) if not stated otherwise. Fermented wheat germ extract, MSC, was produced as described elsewhere (7,9). Water soluble fraction was prepared by dissolving 100 mg MSC in 1 ml water and the insoluble material was removed by centrifugation. The MSC concentration indicated in the experiments was based on the quantity of the dry material before extraction.

Tyrosine phosphorylation of intracellular proteins. Tyrosine phosphorylation experiments were carried out as previously described (12). Briefly, Jurkat or BL-41 cells were harvested from growth medium by centrifugation, washed once in RPMI without FCS and were resuspended at $4x10^7$ cells/ml in RPMI without FCS. Stimulation was initiated by adding 10 μg/ml of anti-T cell receptor (TCR) monoclonal antibody (mAb), OKT3 and 15 μg/ml anti-B cell receptor (BCR) mAb (10A/2) (a kind gift from G. Sármay) to the Jurkat and BL-41 cells, respectively for 1 min or 5 mg/ml of MSC for 10 min, or cells were left unstimulated. Activation was stopped with addition of equal volume of 2X concentrated, ice cold lysis buffer (1X lysis buffer: 50 mM HEPES pH 7.4, 1% Triton X-100, 150 mM NaCl, 20 mM NaF, 5 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethyl-sulfonylfluoride (PMSF) and 10 μg/ml leupeptin). Cells were lysed on ice for 30 min and cleared from nuclear/cytoskeletal components by centrifugation at 12,000 x g for 15 min. Postnuclear supernatants were mixed with equal volumes of 2X SDS sample buffer (13) and loaded onto a 10% SDS polyacrylamide gel. Proteins were then transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked using Tris-buffered saline (TBS) containing 0.05% Tween 20 and 3% cold fish gelatin for 1 h at 37˚C and subsequently probed with anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology Inc., Lake Placid, NY, USA) and rabbit anti-mouse IgG conjugated to horseradish peroxidase (Dako, Carpinteria, CA, USA). Immunoreactive proteins were visualized by an enhanced chemiluminescence (ECL plus) detection system (Amersham, Little Chalfout, UK). Prestained molecular weight marker was purchased from Gibco-BRL (Rockville, MD, USA).

Phosphatase assay rationale. Phosphatase assay was carried out as previously described (14). Briefly, Jurkat cells ($2x10^6$ cells/sample) were lysed for 30 min in ice-cold lysis buffer (1% Triton X-100, 20 mM Tris pH 7.5, 150 mM NaCl, 1.0 mM EDTA) supplemented with 10 μg/ml leupeptin and 1 mM PMSF. Postnuclear supernatants were mixed with 10 μl Sepharose beads (Pharmacia, Peapack, NJ, USA) covalently coupled with anti CD45 mAb, GB3 (15), and incubated for 1 h at 4˚C. The immunoprecipitates were washed twice in lysis buffer and once in phosphatase assay buffer (50 mM HEPES pH 7.0, 100 mM KCl, 0.1% Triton X-100, 1 mM EDTA). The beads were incubated in 45 μl phosphatase assay buffer supplemented with 1 mM dithiothreitol and 1.3 mM para-nitrophenyl-phosphate (pNPP) for 30 min at 37˚C. Fifteen μl of supernatants of the reaction mixtures was transferred into the wells of a 96-well microtiter plate

(Falcon, Bedford, MA, USA) and the amount of the released inorganic phosphate from pNPP was determined according to Ng *et al* (16) using 80 µl of malachite green reagent and a spectrophotometer operating at 650 nm wavelength. Whole cellular phosphatase activity was inhibited with an addition of 10 µM sodium vanadate in the indicated experiments.

Measurement of intracellular Ca^{2+} concentration [Ca^{2+}]i. Jurkat cells were suspended in 10^7 cells/ml concentration in culture medium (RPMI-1640 supplemented with 5% FCS) and incubated with 7.5 µM of Fluo-3AM and 7.5 µM of FuraRed-AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37˚C. Cells were then adjusted to a final concentration of $5x10^5$ cells/ml with the addition of cell culture medium and incubated for 30 min at 37˚C. Cells were washed twice with RPMI and resuspended in fresh cell culture medium at 10^6 cell/ml. Intracellular [Ca^{2+}]i was measured using FACSCalibur (Becton Dickinson, Bedford, MA, USA) and data were presented as the ratio of Fluo-3 and FuraRed fluorescence intensity at 530 and 650 nm, respectively, versus time. In some experiments the influx of Ca^{2+} was inhibited with addition of EGTA in the indicated concentration.

Detection of apoptotic cells. Different cells were treated with either 1 mg/ml MSC, 0,3 µg/ml DMBQ or 6 µg/ml WGA or left unstimulated for 24 h then subjected to DNA content analysis. The cells were then harvested and washed twice with phosphate buffered salt (PBS) containing 0.1% glucose then permeabilized and stained with propidium-iodine (10 µg/ml) in the following solution: 0.1% Triton X-100, 0.1% Na_3-citrate and 0.01% RNase. After incubation for 30 min at room temperature in dark, the cells were analyzed on a FACSCalibur cytofluorimeter (Becton Dickinson, Bedford, MA, USA) using CELLQuest and/or Modfit software programs (Becton Dickinson, Bedford, MA, USA). For Annexin V labeling Jurkat cells were washed twice with PBS and resuspended in binding buffer (0.01 M HEPES, 0.14 M NaCl and 2.5 mM $CaCl_2$). Fluorescein isothiocyanate (FITC) conjugated Annexin V (Pharmingen, San Diego, CA, USA) and propidium-iodine (10 µg/ml) were added to the cells for 15 min in dark, at room temperature. After washing, the cells were analyzed on FACSCalibur cytofluorimeter.

Measurement of cell proliferation. Proliferation assays were carried out in flat-bottomed 96-well tissue culture plates in RPMI supplemented with 5% FCS. The rate of PBMC proliferation was monitored by the incorporation of tritiated thymidine ([^{3}H]TdR) (Amersham, Little Chalfout, UK) into the DNA of the cells in triplicate samples. PBM cells were seeded at 10^5 cells/well with increasing concentrations of MSC and PHA in a final volume of 200 µl and incubated for 72 h. For the last 16 h of culturing, cells were pulsed with 1 µCi/well = 37kBq/well of [^{3}H]TdR, then harvested for scintillation counting.

Analysis of the downregulation of cell surface MHC class I expression. Jurkat, Raji or U937 tumor cells were suspended in $5x10^5$ cells/ml concentration in cell culture medium (RPMI-1640 supplemented with 5% FCS) and incubated in 24-well tissue culture plates with 2 mg/ml MSC or 0.6 µg/ml DMBQ. After 4 h at 37˚C the cells were washed with FACS-buffer (PBS supplemented with 1% FCS and 0.1% NaN_3), and incubated with anti-MHC I monoclonal antibody (TMB6-5, produced in our laboratory) for 1 h at 4˚C followed by goat anti-mouse IgG-FITC for 30 min at 4˚C. The fluorescence intensity was measured on FACSCalibur (Becton Dickinson, Bedford, MA, USA). The MHC I level was calculated as follows: MHC I level=$10^{[(Ch\#TMB6\text{-}5\text{-}Ch\#ctl)/ChD)]}$, where Ch#TMB6-5 = median channel number of anti-MHC I labeled sample; Ch#ctl = median channel number of negative control; ChD = number of channels per decade.

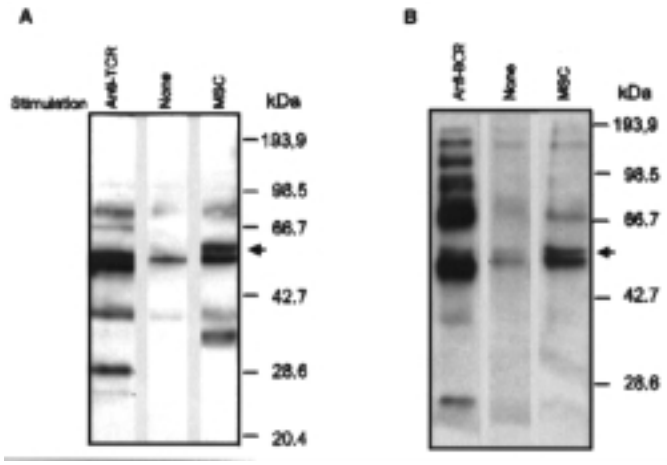

Figure 1. MSC induced protein tyrosine phosphorylation in T and B cell lines. T cell line, Jurkat (A), and B cell line, BL-41 (B), were stimulated for 1 min with 10 µg/ml anti-TCR mAb (OKT3) and 15 µg/ml anti-BCR mAb (10A/2), respectively, or with 5 mg/ml MSC for 10 min or cells were left unstimulated. Postnuclear fraction of the cell lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine mAb, 4G10 followed by rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase. Immunoreactive proteins were visualized by an enhanced chemiluminescence (ECL plus) detection system. Molecular weight markers are presented on the right. The arrow shows the protein that is preferentially phosphorylated by MSC stimulation.

Results

Specific tyrosine phosphorylation in tumor T and B cell lines after MSC treatment. One of the earliest intracellular responses for extracellular signals is the tyrosine phosphorylation of intracellular signal proteins. The newly tyrosine phosphorylated proteins are involved in and many times determine the subsequent biochemical events and therefore the eventual cell response. Short-term exposure of Jurkat cells to the wheat germ extract resulted in reproducible tyrosine phosphorylation of specific proteins with molecular weights of 76, 63 and 38 kDa (Fig. 1A). A protein of 50 kDa was constitutively phosphorylated in the untreated samples, as well. The pattern of MSC induced tyrosine phosphorylation was largely different from the TCR stimulated tyrosine phosphorylation indicating that MSC used distinct signaling pathways from that of TCR stimulation. The B cell line, BL-41 was stimulated with MSC under similar conditions to that of Jurkat cells. In BL-41 an increase in the phosphorylation of several

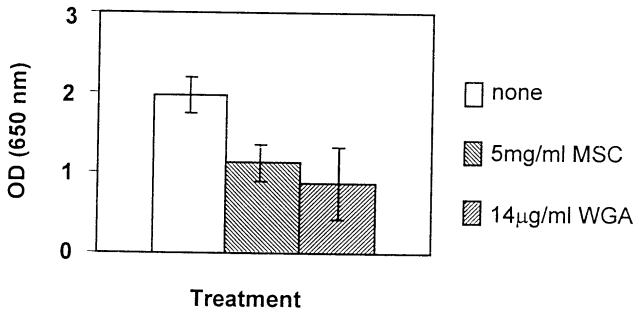

Figure 2. MSC and WGA decreased CD45 tyrosine phosphatase activity. Jurkat cells were treated with 5 mg/ml MSC or 14 μg/ml wheat germ agglutinin for 10 min. The CD45 tyrosine phosphatase was immunoprecipitated on Sepharose beads covalently coupled with anti-CD45 mAb (GB3) and the enzyme activity was measured by the dephosphorylation of the pNPP substrate in duplicates. The amount of the released inorganic phosphate from pNPP was determined using malachite green reagent with spectrophotometer at 650 nm wavelength. (Mean ± SD). The presented experiment is a representative of nine (MSC) and four (WGA) experiments (MSC treatment, p=0.004; WGA treatment, p=0.09; two-tailed, paired t-test, n=9 and 4, respectively).

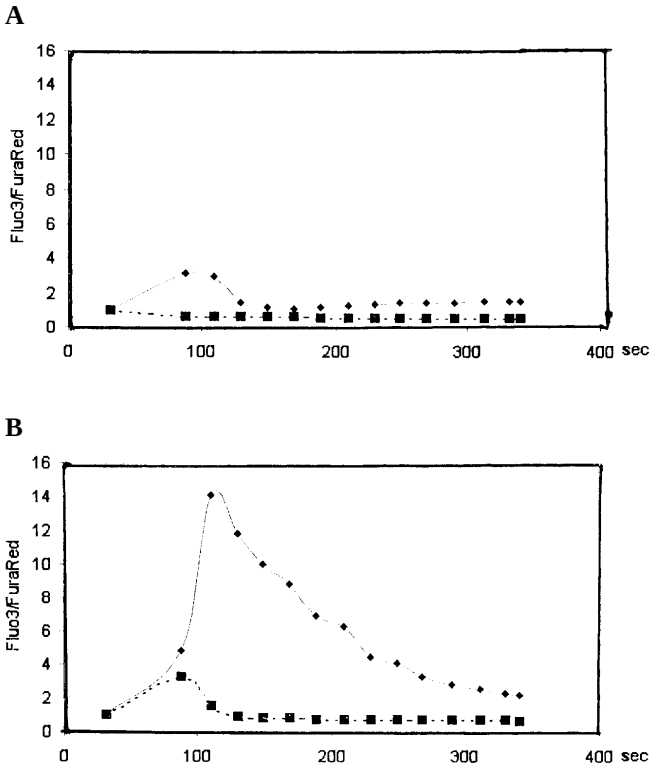

Figure 3. MSC-induced elevation of the intracellular Ca^{2+} concentration is blocked by EGTA. Jurkat cells were loaded with calcium sensitive dyes, Fluo-3AM and Fura Red-AM, then stimulated with 5 mg/ml MSC (A) or 15 μg/ml anti-TCR (OKT3) (B) in the absence (solid line) or presence (dashed line) of 10 mM EGTA. The change in the intracellular Ca^{2+} concentration was analysed with FACS. Data are presented as the ratio of Fluo-3 and FuraRed fluorescence intensity (measured at 530 and 650 nm, respectively) versus time.

proteins was detected upon MSC treatment, compared to the unstimulated control. The pattern of phosphorylation induced by MSC was different from the BCR stimulated (Fig. 1B). A 63 kDa protein was preferentially phosphorylated in both T and B cell tumors indicating a specific role of this protein in MSC stimulated cell response.

CD45 tyrosine phosphatase activity decreases after MSC treatment. CD45, an abundant heavily glycosylated cell surface receptor with intracellular phosphatase activity is expressed on all nucleated leukocytes. It has a pivotal role in leukocyte signaling (17), therefore we analyzed the effect of MSC on the phosphatase activity of CD45. As it is shown in Fig. 2, the phosphatase activity decreased in Jurkat cells upon treatment with wheat germ extract. A similar effect of MSC was detected in B cell line, BL-41 (data not shown). To test whether modulation of phosphatase activity was a direct effect of the lectin present in MSC, we also used purified wheat germ agglutinin (WGA) for cell stimulation. WGA was added to the cells in a concentration that was comparable to its concentration in the MSC dilution used in the experiment (5 mg/ml). WGA treatment resulted in a similar decrease in phosphatase activity than was achieved by MSC treatment (Fig. 2) indicating that the component was responsible for the MSC induced modulation of CD45 activity.

Elevation of intracellular Ca^{2+} concentration in Jurkat cells. Stimulation of Jurkat cells with the wheat germ extract caused an early and transient 3-fold elevation of intracellular Ca^{2+} concentration. This Ca^{2+} entirely came from the extracellular environment, since addition of EGTA, a Ca^{2+} chelator, to the extracellular space blocked the increase of intracellular Ca^{2+} (Fig. 3A). The mechanism and the degree of Ca^{2+} elevation were compared to that induced via the T cell receptor with anti-TCR mAb, OKT3. Anti-TCR triggered a much greater increase (15-fold) in intracellular Ca^{2+} than MSC did and that was composed of the release of intracellular Ca^{2+} (could not be blocked with EGTA) and influx from the extracellular space (blocked with addition of EGTA) (Fig. 3B).

MSC triggers apoptosis in tumor T and B cell lines but does not induce it in healthy peripheral blood mononuclear cells. Incubation of T cell line, Jurkat with MSC at 1 mg/ml concentration for 24 h resulted in apoptosis as demonstrated by cytofluorimetric analysis of the DNA content (Fig. 4A). Tumor B cell line, BL-41 responded to MSC treatment with a similar degree of apoptosis (data not shown). In contrast, MSC treatment of peripheral blood mononuclear cells (PBMC) obtained from healthy donors did not induce apoptosis (Fig. 4A). Early marker of the apoptotic process - e.g. exposure of phosphatydilserine on the outer face of the plasma membrane - was detected on Jurkat cells after 12 h of MSC treatment using Annexin V labeling (Fig. 4B). DMBQ, used in a concentration comparable to its concentration in 1 mg/ml MSC, induced a similar degree of apoptosis in Jurkat to that induced by MSC (Fig. 4A). To determine the role of the early cell responses in the apoptotic process including tyrosine phosphorylation of intracellular substrates and elevation of the intracellular Ca^{2+} concentration, we used Na-vanadate and EGTA to modulate apoptosis. Addition of vanadate to block tyrosine phosphatase activity resulted in an increase of MSC induced apoptosis (Fig. 5). In contrast, the

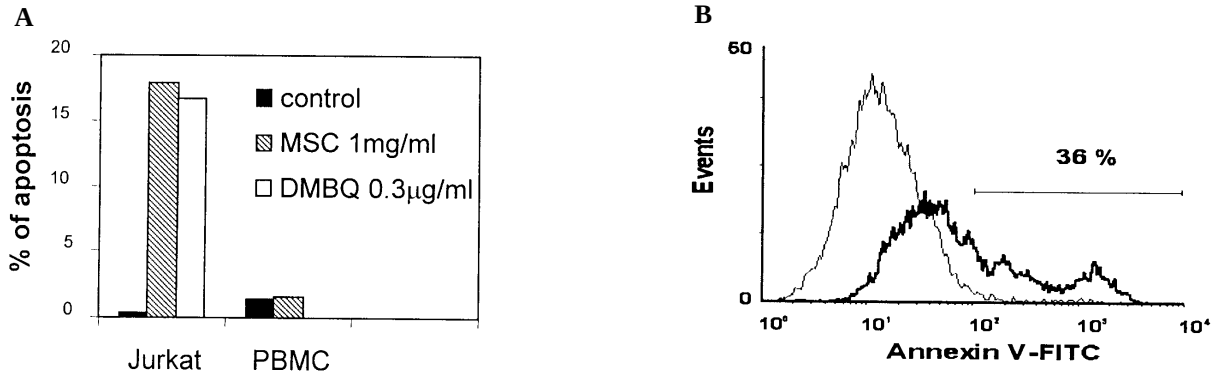

Figure 4. Apoptosis of tumor T cell lines and healthy lymphocytes upon MSC treatment. Jurkat cells were treated with 1 mg/ml MSC or 0.3 μg/ml DMBQ and PBMC were treated with 1 mg/ml MSC for 24 h (A) or Jurkat cells were treated for 12 h (thick line in panel B). Control cells were left unstimulated (black bars in panel A or thin line on panel B). Apoptotic cells were enumerated with the DNA analysis of the 'sub-G_1' population (A) or with staining the cells with FITC labeled Annexin V (B). Representative experiments are shown. The difference between the % of apoptosis in the case of treated and non-treated Jurkat cells was significant (MSC, $p<0.001$, $n=14$; DMBQ, $p<0.05$, $n=3$, using paired, two-tailed t-test). No difference was found for PBMC ($n=2$).

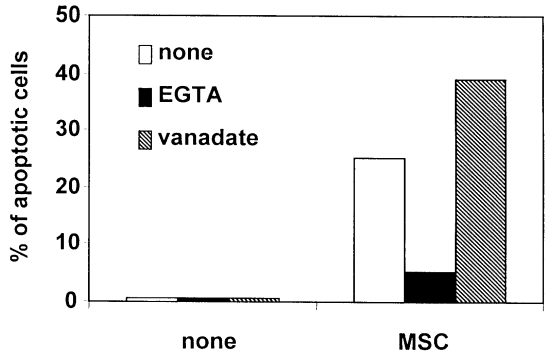

Figure 5. Effect of inhibition of Ca^{2+} influx or tyrosine phosphatase activity on MSC-induced apoptosis. Jurkat cells were incubated with or without MSC in the absence (empty columns) or presence of 2.8 mM EGTA (black columns) or 10 μM vanadate (striped columns). The cells were permeabilized and stained with propidium iodine. The 'sub-G_1' population was determined with analyzing the DNA content with FACS.

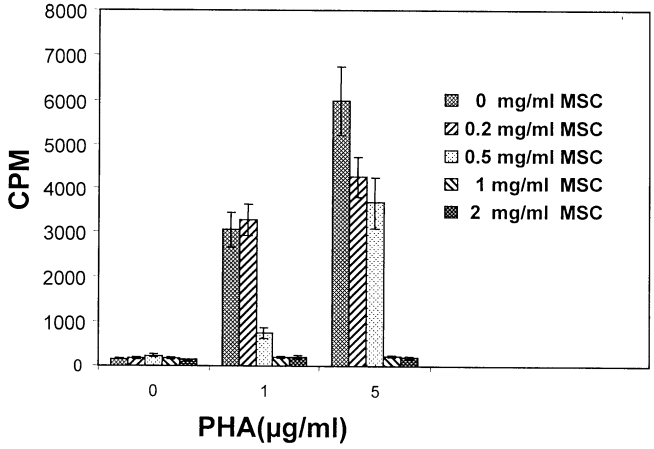

Figure 6. MSC inhibits PHA stimulated proliferation of PBM cells. PBM cells isolated from blood of a healthy donor were stimulated with different concentrations of PHA in the absence or presence of increasing amounts of MSC for 72 h. For the last 16 h, the cultures were pulsed with 1 μCi [^{3}H]TdR then harvested. Incorporation of the isotope into the DNA was measured. Mean ± SD.

presence of EGTA, which blocked the influx of Ca^{2+} from the extracellular space decreased MSC-induced cell death (Fig. 5). Two mutant variants of the Jurkat cell line, J45.01 (deficient in CD45 receptor tyrosine phosphatase) and JCam (deficient in $p56^{lck}$) were also analyzed to prove or exclude the role of CD45 and $p56^{lck}$, two indispensable enzymatic factors of lymphocyte activation (17,18), in MSC-induced apoptosis. The lack of either CD45 or $p56^{lck}$ did not influence the apoptotic process, indicating that these factors may not be directly involved in the MSC-induced apoptosis (data not shown). WGA, a lectin with immunomodulatory properties, present in wheat germ did not induce apoptosis of the tumor cells (data not shown).

The prominent anti-proliferative effect of MSC was demonstrated by the dose-dependent inhibition of PBM proliferation induced by a polyclonal mitogen, phyto-hemagglutinine (PHA) (Fig. 6).

Cell surface expression of MHC class I is downregulated by wheat germ extract treatment. Jurkat T cell line and Raji Burkitt lymphoma cell line were treated with the wheat germ extract for 4 h and the cell surface expression of the MHC class I molecules was analyzed by immunofluorescence.

Fig. 7A shows that MSC treatment of Jurkat cells resulted in a pronounced, 90% decrease of the cell surface MHC class I molecules. Moreover, DMBQ treatment of these cells caused a smaller but still prominent 70% decrease of the cell surface MHC class I as the wheat germ extract did. When Raji B cells were treated with MSC and DMBQ under similar conditions, the loss of MHC class I molecules was lower, 69 and 30%, respectively, compared to the downregulation on Jurkat cells. Similar results were obtained using the $p56^{lck}$ deficient variant of the Jurkat cells. Wheat germ agglutinin treatment did not result in a significant downmodulation of the MHC level in a Jurkat variant (data not shown). Inhibition of intracellular tyrosine phosphatase activity with vanadate resulted in a slightly enhanced downregulation of the MHC

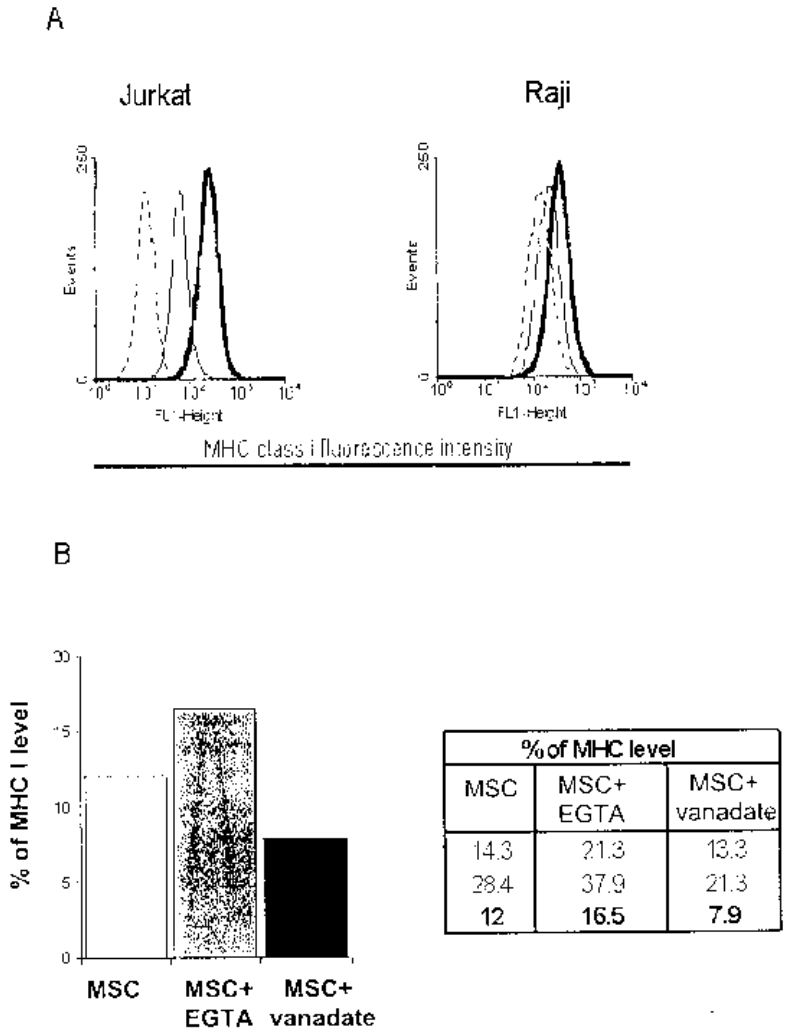

Figure 7. MSC and benzoquinone-treated tumor cells downregulated MHC class I molecules. Jurkat and Raji cells (A) were treated with 2 mg/ml MSC (striped lines) or 0.6 µg/ml DMBQ (thin solid lines) for 4 h at 37˚C or left untreated (thick solid lines). Expression of the MHC class I molecules was analyzed with anti-MHC I monoclonal antibody (TMB6-5) followed by goat anti-mouse IgG-FITC. The fluorescence intensity was measured by FACS. (B), 10 mM EGTA or 10 µM Na-vanadate was added to Jurkat cells in the presence of MSC. The cells were treated and analyzed as described above. The MHC class I level was calculated as described in the Materials and methods and the percentage was calculated as follows: (MHC I level of treated cells/MHC I level of non-treated cells) x 100. The table shows the results of three independent experiments, the data in bold are presented on the graph.

class I on Jurkat cells. In contrast, blocking the MSC-induced Ca^{2+} influx with Ca^{2+} chelator, EGTA produced a smaller downregulation of MHC class I (Fig. 7B). The myelo-monocytic cell line, U937 responded similarly to the MSC treatment in the presence of Ca^{2+} chelator or phosphatase inhibitor as Jurkat T cells did (data not shown).

Discussion

The fermented wheat germ extract, MSC has been shown to possess a strong anti-metastatic feature in animal models and can be used as a supportive therapy in human cancer to reduce metastasis (7,8). In this work we have studied the mechanism by which MSC inhibits metastasis formation.

The wheat germ extract triggered early biochemical events in T and B tumor cell lines. Tyrosine phosphorylation of several intracellular proteins with molecular weight of 76, 63 and 38 kDa was observed upon MSC treatment. The pattern of protein phosphorylation was different from that induced via the TCR or BCR, which are major receptors on T and B lymphocytes, respectively, functioning as specific antigen binding receptors. Stimulation with MSC resulted in the elevation of the intracellular Ca^{2+} concentration by influx of the extracellular Ca^{2+}. The mechanism and the kinetics of the increase of the intracellular Ca^{2+} content was different from that triggered through the TCR. The latter was much higher, longer, and it was composed of Ca^{2+} release from intracellular stores and the influx from the extracellular space. The difference in the tyrosine phosphorylation pattern and the mechanism of the elevation of the intracellular Ca^{2+} induced by MSC and via the T or B cell receptors suggest that MSC induce different signaling pathways from that of TCR or BCR stimulation.

MSC treatment causes prominent apoptosis in lymphoid tumor cells but it does not induce apoptosis of healthy resting mononuclear cells. Moreover, although MSC blocks the proliferation of PBM cells stimulated with PHA, it does not induce apoptosis in PHA stimulated cells (data not shown). It

should be noted that the failure of apoptosis induction in PHA stimulated healthy lymphocytes may be due to the low ratio of intensively proliferating cells in comparison with tumor cell cultures. The early biochemical events, such as tyrosine phosphorylation and elevation of intracellular Ca^{2+} concentration are important elements of the cell response to MSC treatment. Tyrosine phosphorylation of proteins is a result of the concerted activity of kinases and phosphatases. Inhibition of the tyrosine phosphatases with Na-vanadate resulted in an increased apoptosis initiated by MSC indicating that tyrosine phosphorylation of unidentified intracellular substrate(s) plays a role in the MSC-induced apoptosis. The tyrosine phosphatase involved in MSC-induced apoptosis is unlikely identical with CD45, the abundant receptor tyrosine phosphatase on leukocytes because of two reasons: i) The CD45 deficient variant of Jurkat cell line produces a similar apoptotic response to its wild-type counterpart upon MSC treatment; ii) The lectin component of MSC (WGA) inhibits the activity of the CD45 but it does not induce apoptosis of Jurkat cells. The increase of intracellular Ca^{2+} concentration is likely involved in the process of cell death since deprivation of Ca^{2+} from the extracellular environment decreases apoptosis formation.

MSC treatment results in the decrease of the MHC class I protein level on the surface of tumor cells and hence it may expose them to natural killer cell activity. Inhibition of tyrosine phosphatase activity results in an elevated down-regulation of the MHC class I molecules indicating the control of protein tyrosine phosphorylation in this process. In contrast, deprivation of extracellular Ca^{2+} with EGTA blocking MSC triggered Ca^{2+} influx inhibits MHC downregulation.

The involvement of the lymphocyte specific signaling molecules, the non-receptor tyrosine kinase, $p56^{lck}$ and the receptor tyrosine phosphatase, CD45 (17,18) in the MSC triggered cell response has been excluded using $p56^{lck}$ and CD45 deficient variants of the leukemic T cell line, Jurkat. These mutant cells generated a similar cell response to MSC treatment as wild-type Jurkat cells did despite the fact that both $p56^{lck}$ and CD45 have been implicated in lymphocyte apoptosis triggered via the antigen receptors (21,22). These findings supported the view that MSC triggers different signaling pathways from lymphocyte antigen receptors to generate apoptotic response and downmodulation of the cell surface MHC class I proteins.

MSC is a mixture of compounds with potential biological activity. Among these ingredients, benzoquinones are candidates for the anti-tumor effect of the fermented wheat germ extract, because different benzoquinones have been shown to inhibit tumor propagation in previous studies (19,20). Indeed, DMBQ, one of the two benzoquinones present in MSC, produced a similar biological response of the lymphoid tumor cells as MSC did, since it induced the MHC class I downregulation and the apoptosis *in vitro*. However it must be noted that DMBQ has been used in a short time (several hours) experiments in this work. In contrast, when it was applied in long-term *in vivo* studies, DMBQ failed to show the same effect as the whole wheat germ extract did (9). Its inefficiency in long-term, *in vivo* experiments may be explained by the poor solubility and instability in water. We suggest that benzoquinone is a major mediator of the wheat germ extract, and its prolonged biological activity is stabilized by other components of MSC. Alternatively, the fermented wheat germ extract may contain other, yet unidentified component(s) which also play roles in its anti-metastatic effect.

Proliferation, differentiation and cell death are under similar molecular control in all mammalian cells. Cancer cells develop severe defects in the regulation of homeostasis and cell proliferation, including resistance to apoptosis. Any drug that restores the normally operating apoptotic signaling pathways in tumor cells are substantially beneficial in anti-cancer therapy. As we demonstrated herein, the fermented wheat germ extract induces apoptosis in different leukemic human cells, but it does not trigger apoptosis in their healthy, resting counterpart, the peripheral blood mononuclear cells.

Another way for tumors to survive is to evade the defense control of the organism by mimicking normal cells for the survey of the immune system. Natural killer cells, that play an important role in anti-tumor defense, recognize and are blocked by the expression of MHC class I molecules on their target cells (5,23). Consequently, tumor cells develop an effective camouflage by expressing high levels of MHC class I to avoid recognition by NK cells. This is a common characteristic of metastatic tumor cells to avoid NK surveillance (5,24). We demonstrated here that MSC reduces MHC class I level on tumor cells, and therefore it may sensitize human tumor cells against NK killing thus reducing their metastatic activity.

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