Anti-Cancer, anti-Necrotic and Imaging Tumor Marker role of a novel form of Manganese Superoxide Dismutase and its leader peptide

[1]Pica A., [1]Di Santi A., [1]Basile F., [1]Iacobellis F., [2]Borrelli A., [2]Schiattarella A., [2]Mancini R., [2]Mancini A.
(1) University of Naples Federico II, Department of Biological Sciences, Naples, Italy
(2) National Cancer Institute of Naples Pascale, Molecular Biology & Viral Oncogenesis, Naples, Italy.

***Abstract*—**The manganese superoxide dismutase (MnSOD) isolated from a human liposarcoma cell line (LSA) was able to kill cancer cells expressing oestrogen receptors, while it did not have cytotoxic effects on normal cells. The oncotoxic activity of the recombinant MnSOD (rMnSOD) was due, likely, to an increase in the level of oxidants in the tumor cells, which have low levels of catalase and, consequently, reach the threshold of toxicity before normal cells. The effectiveness of the rMnSOD in repairing the damage caused by radical excess was further shown by its topical application on necrotic skin. Together with its oncotoxic activity, the rMnSOD exerts a radioprotective effect on normal cells irradiated with X rays. The rMnSOD is characterized by the presence of a leader peptide, which allows the protein to enter cells: this unique property can be used in the radiodiagnosis of cancer or chemotherapy, conjugating radioactive substances or chemotherapic drugs to the leader peptide of the MnSOD. Compared to traditional chemotherapic agents, the drugs conjugated with the leader peptide of MnSOD can selectively reach and enter only the cancer cells, thus reducing the side effects of the traditional treatments.

***Keywords*—**Anticancer agent, rMnSOD, tumor cells, tumor marker.

I. Introduction

TODAY, chemotherapy is the main therapy for the treatment of cancer, after surgery. Unfortunately all the molecules used as chemotherapic agents have only an antireplicative effect, and in the research in substances having a specific antitumoral activity many efforts are directed to improve their effectiveness on tumor cells. A new approach, based on electrochemotherapy with low frequency (1 Hz) and high frequency (5 kHz) in invasive ductal carcinoma, improves the delivery of chemotherapic drugs, such as cisplatin and bleomycin, into the tumor cells; but the most unpleasant side effect of electrochemotherapy is the muscle contraction due to pulse delivery [1].

The multiple hard adverse effects for patients are among the most negative consequences in contemporary chemotherapy. So, research into effective anti-cancer drugs with no or minor adverse side effects is a big challenge in chemotherapy nowadays.

Sandler et al. (2010) have isolated and cloned a novel human hormone-like protein Nerofe™ (TCApF - Tumor-Cells Apoptosis Factor), a naïve ligand of T1/ST2 receptor that activates caspases 8, 9 and 3-mediated apoptosis, together with the activation of JNKinase and p38 MAPKinase. Application of TCApF and its short derivative TCApFs to acute myeloid leukemia proliferating cells (U937 premyeloid cells), human breast carcinoma (MCF7), human glioblastoma, human prostate cancer, murine mammary adenocarcinoma and human lung cancer proliferating cells inhibits the proliferation *in vitro*. In contrast, TCApF did not affect the healthy cells and was unable to induce apoptosis in non-proliferating cells. The selectivity of TCApF-induced apoptosis is related to the level of T1/ST2 receptor expression [2-3].

A novel human Cytotoxic Cell Activation Factor (CCAcF), has been isolated by Sandler et al. (2010) and demonstrated to be an inhibitor in the proliferation *in vitro* of acute myeloid leukemia cells (U937 premyeloid cells), human melanoma (CAG), human pancreatic cancer cells (Panc1) and human prostate cancer cells (PC3). However, CCAcF does not affect healthy cells [4].

In recent years, the natural antioxidants have gained increasing interest among consumers and the scientific community, particularly those present in fruits and vegetables. Epidemiological studies have demonstrated that frequent intake of fruits and vegetables is associated with a lower risk of cancer and age-related diseases, such as coronary heart diseases. In fact, natural food usually contains dietary antioxidants that can scavenge free radicals [5].

The superoxide and other reactive species, that are free radicals, have been implicated as contributors to tissue injury in a wide range of human diseases, and generation of excess reactive species which can lead to cell death by apoptosis or, if the oxidative stress is severe, by necrosis [6-7].

The enzyme superoxide dismutase (SOD) is believed to be present in all oxygen-metabolizing cells and its physiological function is to provide a defense against the potentially damaging reactivity of the superoxide radical generated by aerobic metabolic reactions [8]. The SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide, which is converted by catalase in oxygen and water [9].

SOD are ubiquitos to all forms of life. Four different types of metal centers have been detected in SODs, dividing this family into Cu/Zn, Ni, Mn, and FeSODs. Cu/ZnSOD is generally homodimeric and is present in diverse locations in different organisms. It is found in the periplasm of bacteria (sod-C),

cytoplasm and chloroplasts of plants, and several compartments such as nucleus, lysosomes, peroxisomes, cytosol (SOD1), and extracellular milieu (SOD3, EC SOD) in animals[10]. Beside these molecules, in the fields of cancer research, the MnSOD has attracted the attention of many researchers, since the expression of the mitochondrial form, MnSOD, has been founded altered in cancer, as well as in other diseases. It is elevated in cancer cells compared to their normal counterparts, including gastric and esophageal [11-12-13], lung [14], colorectal [15] and breast cancer [16]. When there is a higher level of MnSOD in the cancer cells, the aggressiveness of cancer [15] and its metastatic potential [13] are increased, and the prognosis is poor [12]. In some types of tumor (breast and pancreatic cancer) the expression of MnSOD is even decreased.

The onset of cancer usually involves alterations in the control of cell growth and proliferation, DNA damage and reactive oxygen species (ROS) production. The MnSOD plays a role as a tumor suppressive protein, which inhibits cell proliferation and intensifies apoptosis. It influences the activity of some transcription factors, such as activator protein 1 (AP-1), nuclear factor-kappa B (NF-κB) and p53. The MnSOD also protects the normal tissues from chromosomal instability, due to various injuries and causing cancer. Moreover, this enzyme modulates the ROS concentration in cancer cells.

The expression of MnSOD in cancer cells can be altered through mutations in its promoter, epigenetic regulation or genetic polymorphism, which can influence MnSOD transcription, activity or mitochondrial localization. Increased levels of MnSOD were demonstrated to inhibit cancer growth; anyway, in many types of tumor, the MnSOD levels are reduced. As a consequence, the treatment of cancer could benefit from therapies aimed at increasing the expression of this enzyme or by enhancing its specific activity, through the epigenetic activation of its expression or its overexpression using adenoviral vectors.

Another interesting effect of the MnSOD lies in its role as a protective agent for normal cells against the toxic effects of chemotherapic drugs. For example, the most common drugs, adriamycin and anthracyclines, cause a dose-dependent cardiotoxicity, which lead to dilated cardiomyopathy and heart failure, due to an increased production of ROS. The MnSOD, by its enzymatic activity, transforms the ROS into oxygen, thus protecting the heart from the side effects of chemotherapic drugs. Besides the cardiotoxicity, chemotherapy can also cause damage to the brain structure, known as chemobrain, which causes memory loss, decreased reaction time, concentration and attention. One of the mechanisms by which the chemotherapic drugs induce neurotoxicity is, likely, due to the modulation of the ROS detoxifying enzymes, for example the nitration of the MnSOD, whose activity appears to be reduced [17].

Modified isoforms of SOD are present in various tumors and take part in the autocrine mechanisms of cell-growth inhibition. On the other hand, it is well-known that the interaction between tumors and neighboring tissues plays a key role in the process of oncogenesis.

The manganese superoxide dismutase from liposarcoma (LSA-MnSOD) is a tumor protein isolated and sequenced, for the first time, in human liposarcoma cells (LSA) [18].

II. Origin of MnSOD from LSA cells

The LSA cells secrete factors in the culture medium, which can exert a cytotoxic activity on the human mammary tumor cell line MCF-7. In 1991, Mancini et al. [19] successfully cultured and cloned an adipocyte cell line from a human liposarcoma (LSA). In 1999, Mancini et al. [20] managed to have a continuously growing cultured cell line *in vitro*, from ascites fluid belonging to a patient with a poorly-differentiated ovarian adenocarcinoma. The growth of these cells was inhibited by a new regulatory activity, present in the liposarcoma cell line, which arrested their cell cycle in the G1 phase and induced them to apoptosis. The reciprocal action of breast carcinoma cells with cells from the fat tissue surrounding the mammary glands was studied using, as a model *in vitro* system, the interactions between established lines of normal or neoplastic mammary epithelial cells and adipocytes. The liposarcoma-derived cell line (LSA) secreted a factor which was able to kill some tumor-derived mammary epithelial cells, but not the corresponding ***MCF-10*** normal mammary epithelial cells [21]. This factor was the ***LSA-type-MnSOD.*** Immunocytochemical detection of MnSOD in LSA cell, by using a colloidal gold anti MnSOD antibody, showed that MnSOD is located in RER, mitochondria and in secretion vesicles of LSA cells (Fig1).

The identification of the oncotoxic component of the secrete was obtained by a sequence of chromatographic purification steps of the LSA cell culture medium supernatant. For each chromatographic fraction, the cytotoxic activity on MCF-7 cells was tested and the protein content was analyzed by SDS-PAGE. The result was the isolation of a single protein band with a molecular weight of about 30 KDa, which showed an increasing biological activity along with purification steps. The polypeptide was digested with the protease LysC and then sequenced by MALDI-MS/MS. The amino acid sequences were "GELLEAIK" and "GDVTAQIALQPALK", which, in BIOMED, aligned with human MnSOD to 100% and 92.8% respectively. The presence of minor components (14-3-3 proteins) was detected together with the MnSOD in the LSA cell culture medium. In order to assess whether the oncotoxic activity was due to MnSOD or to the minor components, a test of immunodepletion of the putative cytotoxic components was performed, using antisera against human MnSOD, 14-3-3 proteins or MMP-1 as a negative control. The single immunodepleted components were tested on the MCF-7 cells, to evaluate their cytotoxic properties. This experiment demonstrated that the cytotoxic component contained in the LSA cell culture medium is just the MnSOD, which was able to kill cancer cells within 3 hours after application.

III. Production of rMnSOD

The mRNA from growing LSA cells was isolated and a SOD-related cDNA library was made, to find out the complete coding sequence of the component showing

cytotoxic activity. The coding sequence of the MnSOD secreted from LSA cells, compared to that of the human MnSOD sequence, shows a C-T transition at nucleotide 245, producing the substitution of 82-threonine to isoleucine, and a silent transversion at nucleotide 222. So, this protein was sequenced and produced as recombinant form (rMnSOD) [18].

IV. Tumor suppressive activity of rMnSOD

The cytotoxic effect of rMnSOD was tested on a variety of human cell lines: mammary epithelial normal cells (MCF-10) or tumor cells (MCF-7), nonmammary transformed cells (OVCAR-3, MIA PaCa-2, A-375, NCI-28) and nontransformed cells (MRC-5). The LSA-type rMnSOD was used at the final concentration of 1.5 µM. The cytotoxic effect was evaluated by measuring the LDH release from treated cells, indicating cell lysis, and the incorporation of ^{3}H-thymidine, to evaluate the effect of growth inhibition. The results showed that the recombinant protein induced cell lysis and growth inhibition only in neoplastic cell lines.

The cells were immuno-stained with 15 nm colloidal gold–coupled antibody and observed at transmission electron microscope. The MnSOD was detected in the mitochondria, in the Golgi-apparatus and in the secretion vesicles near the plasma membrane: thus, the MnSOD appeared to be released in the medium of LSA cells with an authentic secretion process and not as a consequence of cell death.

In the cultured MCF-7 cells, contrary to MRC-5 cells, a strong increase in the cell concentration of hydrogen peroxide was detected after a 3-hour treatment with rMnSOD, suggesting that cancer cells die after incubation with the recombinant protein, due to their well known low levels of catalase.

These data suggested that the rMnSOD could be used in the therapy of the mammary tumor. The potential of rMnSOD as an anticancer drug was further evaluated *in vivo* in adult female Balb-c-FRIII mice, with mammary adenocarcinoma. While in control mice the primary tumor grew and metastasized in the lungs, in the mice treated with MnSOD, one month after the onset of the administration of the protein, the primary tumor was undetectable and no lesions were found in the lungs.

All the above-described experiments show that the human liposarcoma cell line secrete an oncosuppressive protein, the MnSOD, which, in the recombinant form, could be suitably used as a drug for the treatment of tumors [18].

V. Effects Of rMnSOD On Mammary Epithelial Tumor Cells

In cancer cells – by their well known catalase underproduction, up to 10-100 times lower compared to normal cells – the peroxide cannot be converted in oxygen, so that only cancer cells will reach the threshold of toxicity that will cause apoptotic death. The surprising effect of the rMnSOD is, however, due to its ability, following injection *in vivo*, to penetrate all cells giving off its antioxidant potential, through its enzymatic activity. Both the LSA-MnSOD and the rMnSOD were sequenced and compared to the common mitochondrial form. The difference lies in the presence of a leader peptide, detected only in LSA–MnSOD, thus explaining the unusual characteristic of the protein of entering cells, while the native protein remains confined to the mitochondrial compartment.

To demonstrate that the rMnSOD can exert its cytotoxic activity on tumor cells, expressing oestrogen receptors (ER1), experiments were performed using MCF-7 (human breast cancer) cells.

MCF-7 cells were treated with a 1.5 µM concentration of rMnSOD in the presence or absence of a 10-nM solution of estradiol and only with DMEM supplemented with 10% FCS, the latter were used as a negative control. The MCF-7 cells treated with an excess of estradiol and the negative control cells were viable after 24 hours. The cells that received only rMnSOD had instead all died. These results were confirmed by immunocytochemical analysis. After the treatment, the cells were fixed and the presence of the rMnSOD inside the cells was determined by light microscopy, following immunogold staining. MCF-7 control cells showed the absence of immunostaining in the cytoplasm. MCF-7 cells, after 1 hour of rMnSOD administration, showed the cytoplasm full of silver-enhanced immunogold particles. After 2 hours of rMnSOD administration, the cells showed a labelled cytoplasm and also early necrosis. The MCF-7 cells treated with rMnSOD (1.5 µM) and estradiol showed a large amount of silver-enhanced immunogold particles in the medium outside the cell. The MCF-7 cells treated only with estradiol showed no immunostaining in the cytoplasm. The cytotoxic effect of rMnSOD was evaluated by measuring the LDH released by lysis from treated cells. Approximately 3% and 5% was found respectively in the medium of the control cultures not treated with rMnSOD and of the cultures treated with rMnSOD and estradiol. In the cultures treated with rMnSOD, the released LDH is expressed as a percentage of total LDH measured from cells after lysis following detergent treatment.

Subsequently, to investigate the role that the non-excised 24-residue leader peptide might have in the unusual functions of the rMnSOD, a scrambled peptide with the same amino acid composition but a different sequence, used as control, was synthesized, and cultured MCF-7 cells were separately treated with the FITC-labelled leader peptide or the scrambled sequence for 1 hour at room temperature and then examined by confocal microscopy. Intense cytoplasmic fluorescence was clearly detected in the cells incubated with the leader peptide, demonstrating that the labelled peptide was able to permeate the cells under these conditions. No fluorescence was observed within the negative control cells treated with the FITC-labelled scrambled peptide. In addition, cultured MCF-7 cells were treated with a 1µM oestrogen solution and then incubated with the labelled peptide. Confocal microscopy then showed only a peripheral fluorescence outside the cells, confirming that peptide uptake was inhibited in the presence of oestrogen.

The possible involvement of an apoptotic mechanism was confirmed by inhibition of anti-apoptotic *Bcl2* gene expression detected in tumor cells in the presence of rMnSOD.

So, the internalized rMnSOD may increase the level of oxidants (hydrogen peroxide and hydroxyl radicals) and selectively kills tumor cells, provided that they express oestrogen receptors and low levels of catalase [22].

VI. The rMnSOD topical use

As a further demonstration of the ability of rMnSOD to repair the damage caused by radical excess, its topical formulation was used to treat *Caretta caretta* affected by extended necrotic lesions, which quickly returned to the structural and functional integrity.

Sea turtles in hypothermia are debilitated, move lethargically, float on the surface and show skin necrotic lesions [23]. A loggerhead with necrotic lesions was stranded in the South Adriatic in winter 2005 and subsequently was housed in the Rescue Center of the Zoological Station of Naples. The animal was without (lacked) skin on the dorsal side of the neck and head, showing ischemia, and in some lesions, the dermis and muscle tissue were absent exposing bone; the eyes were covered by palpebral eschar. The end of two fins, lacking skin, connective and muscle tissue, showed exposed bone. The specimen appeared also weakened, hypophagic and anemic. The lesions were treated with topic administrations of rMnSOD, for 4 months, on the necrotic areas. Following topic treatment, the injured tissues were replaced. After about 10 days, the eschar of the neck skin was eliminated and substituted by the new regenerating tissue of a reddish color, due to a local revascularization on the dorsal area of the head. After 80 days, the neck skin was completely regenerated and the head skin regeneration was in progress. After 120 days, the skin was completely regenerated in all the areas including the fins. The rMnSOD topical treatment improved, very likely, oxygen release to tissues (through a mechanism mediated by 2,3 diphosphoglycerate, which facilitates oxygen release from hemoglobin to tissues). The rMnSOD also appeared to induce local revascularization, this is in agreement with its well-known neoangiogenetic properties. The prompt *restitutio ad integrum* of the necrotic tissues was due to the treatment with the rMnSOD, which enzymatically transformed free radicals occurring in the lesion, preventing further necrosis and disruption of the tissue and allowing regeneration of appropriate tissue [24].

VII. rMnSOD As a Radioprotective Agent

The rMnSOD derived from liposarcoma exerts an effect of radioprotection on normal cells and radiosensitization on cancer cells.

The effects of rMnSOD were studied *in vitro*, on irradiated normal and cancer cells, and *in vivo*, on normal mice exposed to lethal doses of 6 Gy. The expression of the antiapoptotic *Aven* gene was investigated.

The first part of the work consisted of clonogenic tests on cells belonging to three cell lines: MRC-5 (normal human lung fibroblasts), MCF-7 (human breast cancer) and LSA (liposarcoma-derived), in the presence or absence of rMnSOD. For each cell line, the cells were divided into four groups, each of them subjected to different treatments. The 1st group was a negative control. The 2nd one was treated only with rMnSOD and the 3rd was only irradiated, to evaluate the effect of the single treatments on clonogenic survival. The 4th group was irradiated and treated with rMnSOD, to determine the result of the two combined treatments.

In normal cells (MRC-5 cell line), the sole rMnSOD showed low toxicity, the sole irradiation led to 90 % of cell death, while 80 % of these cells, with the combination of the two treatments, did not die, thus showing the radioprotective effect of rMnSOD on normal cells.

In MCF-7 cells, both the sole treatment with rMnSOD and the sole irradiation led to cell death, while the same effect was obtained with the combination of rMnSOD, in a concentration lower than that required when used alone, and X-rays, at a dose lower than that required to kill cancer cells only with irradiation.

In the LSA cells, which show a well known radioresistance, neither the sole rMnSOD nor the sole irradiation caused damage of the cells: instead, the two combined treatments caused a rapid mortality. The results on MCF-7 and LSA cells showed the radiosensitizing effect of rMnSOD on tumor cells.

The second goal was the study of the effects of rMnSOD *in vivo*, in healthy female mice exposed to X-rays, with the monitoring of their survival and the histological analysis of heart, cervix uterus, liver and kidney. In the organs of the control animals, irradiated and then subjected to daily doses of PBS, signs of necrosis were evident and the rMnSOD absent; these mice died 6-7 days after irradiation. The organs from animals irradiated and then subjected to daily doses of rMnSOD did not appear radio-damaged, and the rMnSOD was detected in the interstitial spaces of the tissues; 80% of these mice were alive 30 days after irradiation. Hence, the radioprotective effect of rMnSOD was demonstrated *in vivo* too: it was hypothesized that the rMnSOD protects the mitochondria, by maintaining their integrity.

The third objective consisted of the molecular analysis of the *Aven* gene, by means of RNA extraction, reverse transcription and PCR amplification. The *Aven* gene was constitutively activated in healthy mice which did not receive any treatment, neither irradiation nor injections of rMnSOD. The mice irradiated and not treated with rMnSOD showed the complete switch off of the *Aven* gene and the structural degeneration of their organs. On the contrary, the mice irradiated and then treated with rMnSOD showed a partial inactivation of the *Aven* gene 7 days after irradiation and its full expression 14 days after irradiation. The rMnSOD inhibits the inactivation of the *Aven* gene, whose product binds the cytochrome c released by the apoptotic mitochondria. When the cytochrome c is released in large amounts, the *Aven* gene is inactivated and the caspase activation is helped, leading to apoptosis. It was supposed that the radioprotective effect of the rMnSOD is exerted through the inhibition of the inactivation of the *Aven* gene, corresponding to inhibition of apoptosis [25].

VIII. Structure Of MnSOD: Discovery Of The Leader Peptide

The novel isoform of manganese superoxide dismutase (LSA-type MnSOD) not only exerts the enzymatic activity common to all SODs, with the transformation of free radicals into hydrogen peroxide, but also shows peculiar structural and functional properties, compared to the corresponding native MnSOD molecule expressed in the human myeloid leukemia cell line U937 (Swiss Prot. Code

P04179). The LSA-type MnSOD, in fact, is secreted by LSA cells, contrary to the native MnSOD, which is located in the mitochondrial matrix. The SDS-PAGE demonstrated that the molecular weight of the LSA type MnSOD is higher than of human native MnSOD.

Human T7-tagged rMnSOD was purified from *E. coli* cell extract by a single immunoaffinity chromatography and aliquots were subjected to SDS-PAGE to examine the purity grade. Fractions containing a single protein band were gathered and structurally characterized. Purified native and carboxyamidomethylated rMnSOD samples were first analyzed by electrospray mass spectrometry (ESMS). The native protein exhibited a molecular mass of 26662.7 ± 1.2 Da. Following alkylation of the protein, the molecular mass increased to 26895.6 ± 0.9 Da, corresponding to four carboxyamidomethyl groups incorporated. By means of the automated Edman degradation, the N-terminal sequence of the first 20 amino acids of rMnSOD, confirmed the presence of the total T7 tag, made up of 15 amino acids, at the N terminus of the protein. The remaining 5 residues coincided with the leader peptide of human MnSOD, pointing out the absence of proteolysis. The human MnSOD sequence reported in the Swiss Prot database (code: P04179) comprises of 221 residues (without the N-terminal Met), including a 24-residue leader peptide for mitochondrial import, and contains 3 Cys residues. The presence of this sequence leads to a molecular mass of 24722.1, which increases to 26247.6 Da, when the 15-residue peptide is included (236 residues). Aliquots of the reduced and carboxyamidomethylated recombinant protein were individually digested with 3 enzymes and the resulting peptide mixtures directly analyzed by MALDI mass spectrometry, showing a nearly complete alignment of the protein sequence from the N-terminal amino acid to residue 236. The data obtained with ESMS and MALDI mass spectra suggested that the peptide extension might have been located at the C-terminus. Moreover, after endoprotease V8 digestion, 2 mass signals at m/z 1431.6 and 1874.9 in the MALDI spectra were obtained. They could not be assigned to any peptide within the rMnSOD sequence, because, very likely, these fragments might contain the additional amino acid residues. After digestion, the fragments were separated by HPLC and each fraction was manually collected and directly analyzed by MALDI mass spectrometry. The following step was the determination of the amino acid sequence of the fraction containing the peptide with a mass value of 1431.6 Da, whose sequence aligned with the C-terminal peptide of rMnSOD from 231 to 236 plus the additional 5-residue sequence Asn-Lys-Asn-Ser-Cys. This extension contained the fourth cysteine residue predicted previously by ESMS analysis. Moreover, the experimental value determined by ESMS was in accord with the molecular mass of the rMnSOD, calculated after inclusion of the C-terminal extension (26666.2 Da). The presence of the leader peptide at the N-terminus of the protein was confirmed by the peptide mapping of the LSA-type MnSOD with the C-terminal end of the LSA secreted protein not showing the additional 5-amino acid peptide.

The sequencing of cDNA was used to determine the whole coding region of the gene for rMnSOD, in which a single point mutation had occurred after the codon corresponding to Lys 221 of the protein sequence, generating a new stretch of 5 codons.

The rMnSOD is characterized by a secondary structure made up of 50% of α-helix, 15% of β-sheet and 15% of turns. The melting temperature (Tm) value is 59°C [24].

IX. The Leader Peptide As Molecular Carrier

The use of antireplicative drugs in cancer therapy is subjected to many limiting properties: for example, they are not specific for cancer cells and exert their cytotoxic activity not only on tumor cells, but, unfortunately, also on normal cells.

The rMnSOD is able to enter tumor cells, due to the presence of the leader peptide, thus becoming suitable as a carrier which can transport antitumoral drugs directly into their target cells.

So, the leader peptide has been synthesized (rMnSOD-Lp) and injected *in vitro* and *in vivo*, thus demonstrating its ability to enter cells: these experiments highlighted its role as a molecular carrier.

In order to evaluate the effective carrier capacity of the rMnSOD, experiments were performed on MCF-7 (human mammary cancer cells), MCF-10 (human normal mammary epithelial cells), MiaPaCa (human pancreatic carcinoma), A2780 (human ovarian cancer cells) and MRC-5 (human normal lung fibroblasts). These cells were treated in the presence of 92 μM of cisplatin or 92 μM of rMnSOD-Lp-CC (rMnSOD leader peptide conjugated with 11.1 μg of cisplatin), against controls which were incubated in the presence of the sole peptide (92 μM) in the absence of cisplatin. The amount of platin after incubation, measured by atomic absorbance spectrophotometry, in the cells treated with rMnSOD-Lp-CC was 7 to 80 times greater than that found in cells treated with the sole cisplatin.

Cytotoxic assays were performed for all the cell lines by adding individually rMnSOD-Lp-CC, rMnSOD-Lp or cisplatin at a final concentration ranging from 0.06 to 1 μM. The results showed that the sole leader peptide did not exert any toxic effect, while the rMnSOD-Lp-CC was more cytotoxic than the cisplatin alone.

To reveal the apoptotic effect of the rMnSOD-Lp-CC, the expression of the pro-apoptotic *Bax* gene was evaluated by PCR analysis. A strong *Bax* transcript band was found in MCF-7, A2780 and MiaPaCa cells treated with rMnSOD-Lp-CC, as well as in those treated with cisplatin alone, while the *Bax* gene expression is totally absent in the control cells and in those treated with the sole leader peptide. Low levels of expression were found in MCF-10 and MRC-5 cells, without significant differences between the cells treated with rMnSOD-Lp-CC and those treated with the sole cisplatin. The activation of the apoptotic pathway by the rMnSOD-Lp-CC was confirmed evaluating the expression of Erk. The ERK protein was quantified by Western blotting, after the treatment of the MCF-7 cell in the presence or absence of rMnSOD-Lp-CC and compared to cisplatin or leader peptide alone. It was shown that the rMnSOD-Lp-CC and the cisplatin alone induce the *Erk* activation, contrary to the leader peptide alone, so the rMnSOD-Lp-CC has an apoptotic function [26].

X. The rMnSOD and its Leader Peptide In Radiodiagnostic

For biodistribution studies, normal animals or animals bearing A2780 cell xenografts derived from a human ovarian adenocarcinoma were injected i.p. with 4 μg of ^{125}I-labelled protein. The treated animals showed a higher percentage in the relative amount of radioactivity in the organs. *In vivo* localization of rMnSOD was monitored by treating a 12-year-old female dog weighing 23 kg suffering from breast cancer with 500 μCi of ^{131}I-labelled rMnSOD. The protein could selectively reach and localize at the tumor site even at an early stage after injection, as shown by the scintigraphic images of the abdomen (ventral view) obtained 3 and 24 hr after injection.

The synthetic leader sequence of rMnSOD labelled with ^{68}Ga was injected into a dog bearing a spontaneous mammary tumor, followed by PET examination 2 hours later. Sagittal PET images showed a high concentration of the radiolabelled peptide in the tumor. These data confirmed that the peptide has the capacity of a molecular carrier and it can be used to transport several drugs and molecules into the tumor cells. Nevertheless, the leader peptide can be used in the radiodiagnosis, as it was shown to unambiguously localize the site of the tumor [5]. Further studies about the carrier function of the leader peptide were performed by conjugation of this peptide to radioactive ^{68}Ga and its subsequent injection into animals affected by spontaneous mammary tumors. PET analysis, performed two hours after injection in animals (dogs and cats), demonstrated internalization of the synthetic construct in the tumor and in its metastasis, thus allowing a more accurate identification in the body [18, 22].

XI. Conclusions

THE functional studies on the rMnSOD showed that it plays a role as a specific, targeted cytotoxic agent only on cancer cells. Its leader peptide was demonstrated to be a good carrier of cytostatic drugs by allowing them to enter in higher amount the cancer cells and induce apoptotic death, so that lower amounts of chemotherapic drugs can be used, thus reducing the side effects. Moreover the administration of rMnSOD results in a protective effect on normal cells and reduces the secondary side effects that usually accompany the chemotherapic treatment. The innovative result of the above studies is that the leader peptide, as carrier of cytostatic drugs, transforms the generic antireplicative activity of the chemotherapic drug in a specific and selective anti-tumor activity, greatly improving patient survival.

References

[1] **Z. Shankayi, S. M. P. Firoozabadi, L. Towhidi and E. Raeisi.** Antitumor Efficiency of ElectroChemoTherapy by High and Low Frequency and Repetitive Therapy in Treatment of Invasive Ductall Carcinoma in Balb/c Mice. Recent Advances in Clinical Medicine; 2010; 228-232. ISSN: 1790-5125 ISBN: 978-960-474-165-6

[2] **U. Sandler, O. Devary, O. Braitbard, J. Ohana, G. Kass and Y. Devary.** A Novel Human Hormone-peptide NEROFE™ with Strong anti Cancer Activity. Recent Advances in Clinical Medicine; 2010; 156-161. ISSN: 1790-5125 ISBN: 978-960-474-165-6

[3] **U. Sandler, O. Devary, O. Braitbard, A. M. Rubinstein, Z. Y. Friedman and Y. Devary.** A Novel Human Hormone-peptide NEROFE Selectively Induces Apoptosis in Cancer Cells through the T1/ST2 Receptor. Recent Advances in Clinical Medicine; 2010; 162-167. ISSN: 1790-5125 ISBN: 978-960-474-165-6.

[4] **T. Sandler, O. Braitbard, J. Ohana, G. Kass and O. Devary.** A Novel Human Cytotoxic Cell Activation Factor with anti-Cancer Activity. Recent Advances in Clinical Medicine; 2010; 168-173. ISSN: 1790-5125 ISBN: 978-960-474-165-6.

[5] **X. Jia, C. Zhao and Y. Chen.** The in vitro and vivo antioxidant activities of various extracts from Artemisisa Selengensis Turcz. Recent Advances in Clinical Medicine; 2010; 261-266. ISSN: 1790-5125 ISBN: 978-960-474-165-6

[6] **B. Halliwell and J. M. C. Gutteridge**. Free Radicals in Biology and Medicine, 3rd edition. Oxford University Press, UK; 1999.

[7] **M. B. Hampton, B. Fadeel and S. Orrenius**. Redox regulation of the caspases during apoptosis. Ann. NY Acad. Sci.; 1998; 854, 328-335.

[8] **J. M. McCord, B. B. Keele and I. Fridovich.**.An enzyme based theory of obbligate anaerobiosis: the physiological function of superoxide dismutase. Proc. Nati. Acad. Sci. U.S.A.; 1971; 68: 1024-1027.

[9] **D. Klug and J. Rabini.**. A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. J Biol Chem; 1972; 247:4839–4842.

[10] **B. Halliwell**. A super way to kill cancer cells? Nature Medicine; 2000; 6 (10).

[11] **R. Izutani, S. Asano, M. Imano, D. Kuroda, M. Kato and H. Ohyanagi.** Expression of manganese superoxide dismutase in esophageal and gastric cancers. J Gastroenterol; 1998; 33:816–822.

[12] **A. M. L. Janssen, C. B. Bosman, W. Van Duijn, M. M. Oostendorp-Van de Ruit, F. J. G. M. Kubben, G. Griffioen, B. B. H. W. Lamers, J. H. J. M. Van Krieken, C. J. H. Van de Velde and H. W. Verspaget.** Superoxide dismutases in gastric and esophageal cancer and the prognostic impact in gastric cancer. Clin Cancer Res; 2000; 6:3183–3192.

[13] **M. Malafa, J. Margenthaler, B. Webb, L. Neitzel and M. Christophersen**. MnSOD expression is increased in metastatic gastric cancer. J Surg Res; 2000; 88:130–134.

[14] **J. C-m Ho, S. Zheng, S. A. A. Comhair, C. Farver and S. C. Erzurum.** Differential expression of manganese superoxide dismutase and catalase in lung cancer. Cancer Res; 2001; 61:8578–8585.

[15] **Y. Toh, S. Kuninaka, T. Oshiro, Y. Ikeda, H. Nakashima, H. Baba, S. Kohnoe, T. Okamura, M. Mori and K. Sugimachi.** Overexpression of manganese superoxide dismutase mRNA may correlate with aggressiveness in gastric an colorectal adenocarcinomas. Int J Oncol; 2000; 17:107–112.

[16] **E. Tsanou, E. Ioachim, E. Briasoulis, K. Damala, A. Charchanti, V. Karavasilis, N. Pavlidis and N. J. Agnantis**. Immunohistochemical expression of superoxide dismutase (MnSOD) anti-oxidant enzyme in invasive breast carcinoma. Histol Histopathol; 2004; 19:807–813.

[17] **A. K. Holley, S. K. Dhar**, **Y. Xu and D. K. St. Clair.** Manganese superoxide dismutase: beyond life and death. Springer-Verlag; 2010. DOI 10.1007/s00726-010-0600-9.

[18] **A. Mancini, A. Borrelli, A. Schiattarella, S. Fasano, P. Sehr, A. Occhiello, A. Pica, M. Tommasino, J. P. F. Nuesch and J. Rommelaere.** Tumor suppressive activity of a novel isoform of

Mn-Superoxide-dismutase released by a human liposarcoma cell line. Int Jou of Cancer; 2006; 119: 932-943.

[19] A. Mancini, C. Garbi, F. D'Armiento, A. Borrelli and F. S. Ambesi-Impiombato. Culture and cloning of an adipocytes cell line from a human liposarcoma. Boll Ist Tum Napoli; 1991; 38: 43-49.

[20] A. Mancini, A. Borelli, S. Formisano, M. T. Masucci, A. Maffeo, G. Perla, L. De Martino, N. Bevilacqua, G. Botti and T. Maggino. Establishment and growth regulation of a novel ovarian cancer cell line from a poorly differentiated adenocarcinoma: proposal for a new treatment. Eur J Gynaecol Oncol; 1999; 1: 43–9.

[21] A. Mancini, A. Borrelli, M. T. Masucci, A. Schiattarella, S. Filice, J. Rashan and T. Maggino. A conditioned medium from a human liposarcoma derived cell line induces p53-dependent apoptosis in several tumor cell lines. Oncol Rep; 2000; 7: 629–37.

[22] A. Mancini, A. Borrelli, A. Schiattarella, L. Aloj, M. Aurilio, F. Morelli, A. Pica, A. Occhiello, R. Lorizio, R. Mancini, A. Sica, L. Mazzarella, F. Sica, P. Grieco, E. Novellino, D. Pagnozzi, P. Pucci and J. Rommelaere. Biophysical and biochemical characterization of a liposarcoma-derived recombinant MnSOD protein acting as an anticancer agent. Int. J. Cancer; 2008; 123: 2684-2695.

[23] B . E. Witherington and L. M. Ehrhart. Hypothermic stunning and mortality of marine turtles in the Indian River Lagoon System, Florida. Copeia; 1989; 696-703

[24] A. Occhiello, F. Bentivegna, A. Borrelli, A. Schiattarella, A. Mancini and A. Pica. Skin necrosis in sea turtle cold stunning: regeneration following rMnSOD topic treatment in a specimen of *Caretta caretta*. Comp. Clin. Pathol.; 2009; 18: 365–369.

[25] A. Borrelli, A. Schiattarella, R. Mancini, B. Morrica, V. Cerciello, M. Mormile, V. d'Alesio, L. Bottalico, F. Morelli, M. D'Armiento, F. P. D'Armiento and A. Mancini. A recombinant MnSOD is radioprotective for normal cells and radiosensitizing for tumor cells. Free Radical Biology & Medicine; 2009; 46: 110–116.

[26] A. Borrelli, A. Schiattarella, R. Mancini, F. Morelli, C. Capasso, V. De Luca, E. Gori and A. Mancini. The leader peptide of a human rec. MnSOD as molecular carrier which delivers high amounts of cisplatin into tumor cells inducing a fast apoptosis in vitro (**Int.J. Cancer-2010-UICC).**

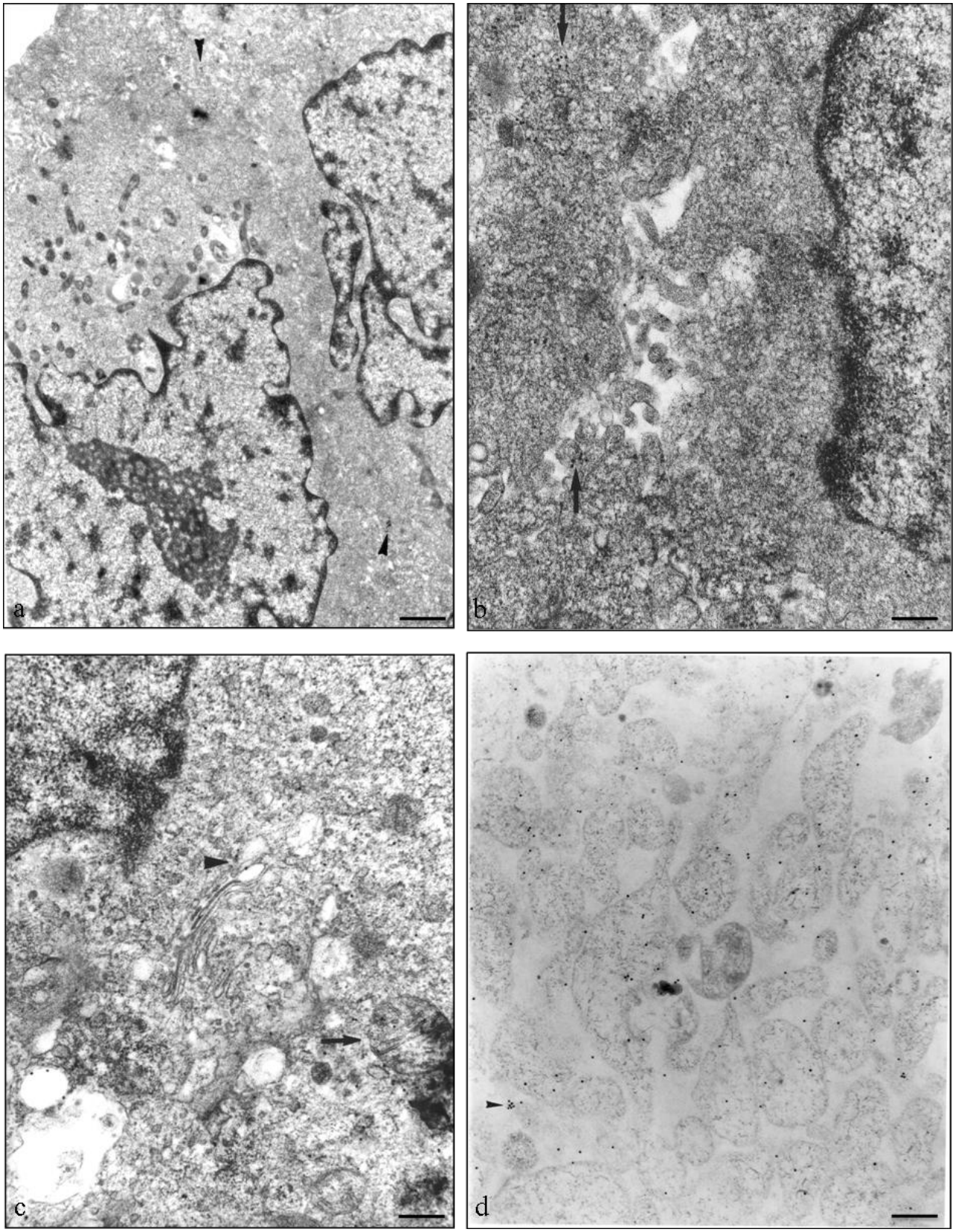

Fig1. Transmission electron microscopy of LSA cells. Immunogold method: 15 nm colloidal gold antibody.
Fig1a. Low magnification of three LSA cells showing the contact among them and the secretion vesicles containing LSA-Mn-SOD (arrow head) Scale bar (in D): 150 nm.
Fig1b. Note the clustered colloidal gold particles in the secretion vesicles and in RER of LSA cell (arrows). Scale bar (in D): 450 nm.
Fig1c. Detail of LSA cell showing LSA-Mn-SOD (demonstrated by the colloidal gold particles) in Golgi vesicles (arrow head) and in mitochondria too (arrow). Scale bar (in D): 450 nm.
Fig1d. TEM section of LSA cell without osmium tetroxide to preserve immunogenicity. Immunoreaction for LSA-Mn-SOD revealed by 15 nm colloidal gold. Note the abundance of colloidal gold particles in the secretion vesicles. Scale bar: 450 nm.