[Frontiers in Bioscience E5, 974-981, June 1, 2013]

Electroporation promotes HtrA1 uptake and in a mouse model of mesothelioma

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1. ABSTRACT

There is not a consensus on the best therapeutic approach to mesothelioma and the prognosis is still dismal. We have recently demonstrated that HtrA1 is a potential therapeutic target in mesothelioma cells. In this manuscript we describe that electroporation in a mouse mesothelioma xenograft was able to facilitate the expression of exogenous HtrA1 injected intra-lesionally in the tumors and to increase the penetration in the neoplastic cells of cisplatin given intra-peritoneally. Indeed, HtrA1 over-expression caused a significant slowing down of tumor growth; moreover, cisplatin efficacy in reducing tumor mass was amplified by electroporation and this phenomenon was even more significant when combining the electroporation of cisplatin and HtrA1. Considering that a substantial number of mesothelioma patients develop early local recurrence, even with radical resection combined with aggressive chemo- and radiotherapy, this multi-modality approach could be very effective in improving local tumor control after surgery. The identification of effective combination coupled with the development of novel equipments and electrodes will be instrumental in planning the translation of these results to humans as per correct laboratory-clinical interface.

2. INTRODUCTION

The incidence of malignant mesothelioma (MM) is significantly raising in western countries (1-4). The pathogenesis of MM, as well as the molecular mechanisms causing its tumor progression are rather complex and largely unknown (5,6). The association between MM and asbestos exposure is widely accepted, primarily in the amphibole forms (needle like fibers), crodidolite and amosite (7,8). Nevertheless, it has been shown that even if 80% of the people with MM had exposure to asbestos, only 10% of the individuals exposed to that carcinogen develop a MM (9), thus implying that other independent factors could be involved. Finally, a link between MM and the simian virus 40 (SV40) has been highlighted by several investigators, showing that mesothelioma cells are selectively positive for SV40 while the adjacent tissues do not express viral sequences (10-12). The treatment of MM is a major challenge. Despite the adoption of newly developed radiotherapic and chemotherapic regimens, the prognosis remains dismal and only modest improvements have been obtained thus far. In this scenario, novel strategies to improve the efficacy of chemotherapy drugs are much warranted. Electroporation therapy (EP) is a treatment modality that uses brief, high-intensity, pulsed electrical currents to enhance the delivery of

chemotherapeutic agents, vaccines and genes to tumour cells. This method was initially used to transfect bacterial cells with plasmids, and subsequently exploited to produce monoclonal antibodies through fusion of eukaryotic cells (13). Later, researchers realized that EP might enhance the transport of drugs and genes through the cytoplasmic membrane by exposing animal cells in culture and plant protoplasts to non-cytotoxic electric pulses (14,15). Moreover, EP has been proven to be very effective at enhancing the *in vitro* cytotoxicity of anticancer molecules, which in the case of bleomycin, led to an enhancement of 300-700 fold (16). Only a few clinical trials have been conducted in animals and humans over the past ten years, since the first phase I-II EP trial was performed (17). In these cohorts of patients different voltages, waveforms and delivery modes (i.e. single pulses versus bursts) were tested (18-28). The results of some studies have shown that electric pulses are capable of driving plasmid into muscle cells resulting in DNA protection from extracellular endonucleases and increased gene expression in rodent and canine models (29,30). The objective of this study was to evaluate whether EP could increase the efficacy of the serine protease HtrA1 cDNA trasfection *in vivo* on a mouse MM xenograft model, alone or in combination chemotherapy with cisplatin (CDDP). The rationale of this study was based on the observation that HtrA1 (a member of the High Temperature requirement A) acts as an endogenous modulator of CCDP-induced cytotoxicity and has been described as a prognostic parameter for MM, thus suggesting this serine protease as a possible molecular target for the treatment of MM (31,32).

3. MATERIALS AND METHODS

3.1. Reagents

The human mesothelioma cell line MSTO-211H was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured as monolayers in flasks using ATCC complete growth medium in a humidified atmosphere containing 5% CO_2 at 37°C.

The *HtrA1* cDNA was cloned into pcDNA3-T7Tag vector (Invitrogen, Groningen, The Netherlands) between the BamHI and EcoRI sites as previously described (33). The rabbit polyclonal immune serum against human HtrA1 was produced by immunizing rabbits with a purified bacterially-expressed glutathione-S-transferase (GST)-HtrA1 (aa 363-480) fusion protein, as described (33).

3.2. Electroporation

The Chemipulse (34) is built up by a toroidal core transformer generating a roughly rectangular pulse which is split in two halves that are sequentially driven to obtain a biphasic pulse. The pulses are not singularly produced but are created in bursts of eight, thus reducing the treatment time and the overall patient morbidity. The equipment allows to choose among a broad range of voltages (from 100 to 2400 V) with sequential increases of 100 V. The standard train is set to 8 pulses of 50 + 50 μs duration with one ms interpulse interval resulting in a total burst duration of 7.1 ms. The pulse repetition frequency is 1 Hz. *In vivo*, sequential bursts of 8 biphasic pulses lasting 50+50 μs were applied to tumor nodules at a voltage of 1300 V/cm using autoclavable caliper electrodes. Adherence of the electrodes to the lesion was maximized using an electroconductive gel.

3.3. Animals and treatment

Male nude mice (6-8 weeks old; weight 18-25 g) were obtained from Harlan Laboratories (Milan, Italy). Mice were housed in the animal facility of the Regina Elena Cancer Institute for a week before each experiment; animals had *ad libitum* water and food. Each experiment used 7 mice per group and was repeated twice to confirm the obtained data. All procedures involving mice and their care were done according to institutional guidelines in compliance with National and International laws and policies (European Economic Community Council Directive 86/109, OJL 358, Dec. 1, 1987, and with the NIH Guide for the Care and Use of Laboratory Animals). The ethical committee of the Cancer Institute approved all the experimental protocols that were done in accordance with Italian law (116/92) and with the Guide for the Care and Use of Laboratory Animals.

$1 \cdot 10^7$ mesothelioma cells in 0.3 mL of their complete medium were injected in the dorsum of mice. One week later, animals were randomly allocated to one of the following groups: (a) control; (b) HtrA1 plasmid (9μg) plus EP; (c) CDDP (10mg/kg intraperitoneally) alone; (d) CDDP (10 mg/kg intraperitoneally) plus EP; (d) combination of CDDP and HtrA1 plasmid plus EP following the schedules above. EP of the plasmid was performed at day 1 and day 8 of the experiment. Tumors were measured with Vernier caliper every fourth day. Experiments were closed at the twenty-fourth day from the starting of treatment. Tumor size was assessed using the formula (π x long axis x short axis x depht) / 6 and expressed in mm^3.

3.4. Tissue collection and histology

Tumour xenografts of the animals were collected en-block at 24 days, divided in two halves. One half was immediately frozen for protein analysis; the second half was fixed in paraformaldeyde and included in paraffin. We performed histological analysis, using Haematoxylin/Eosin and Haematoxylin/Van Gieson staining. For immunohistochemistry, sections from each specimen were cut at 5 μm, mounted on glass and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antibody dilution. Endogenous peroxidase activity was blocked by 5% hydrogen peroxide. Tissue sections were heated twice in a microwave oven for 5 min each at 700 W in citrate buffer (pH 6) and then processed with the standard streptavidin-biotin-immunoperoxidase method (DAKO Universal Kit, DAKO Corp., Carpinteria, CA, USA). Anti-HtrA1 polyclonal antibody was used as previously described (35,36). Diaminobenzidine was used as the final chromogen, and hematoxylin as the nuclear counterstain. Negative control experiments for each tissue section were performed in the absence of the primary

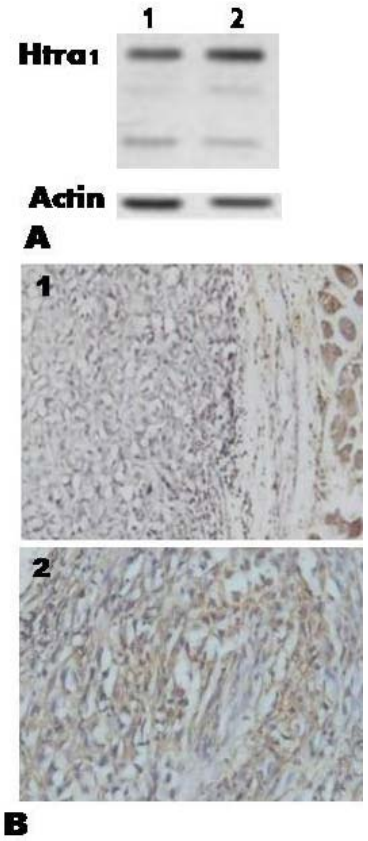


Figure 1. Panel A: Western blot analysis of HtrA1 protein expression in control tumours (line 1) and in tumours electroporated with HtrA1 expression vector (line 2). Panel B: Immunohistochemical analysis of HtrA1 protein expression in control tumours (image 1) and in tumours electroporated with HtrA1 expression vector (image 2) (original magnification X20).

antibody. Positive controls included in each experiment consisted of tissue previously shown to express the antigen of interest. All samples were processed in the same run as one batch.

3.5. Western blotting

Cell lysates were prepared by treating cells with ice-cold lysis buffer (20 mM Tris pH 8.0, 1% NP40, 10% glycerol, 137 mM NaCl, 10 mM EDTA pH 8.0, and Roche Applied Science protease inhibitor cocktail "Complete") for 20 minutes, followed by high speed centrifugation at 4°C for 15 minutes. Proteins (50 µg) were separated on 12% SDS-PAGE gels and then transferred on polyvinylidene difluoride membrane. Membranes were incubated with HtrA1 polyclonal antibody (37) diluted 1:100 or with beta-actin antibody (clone AC-15, Sigma Aldrich, Saint Louis, USA), diluted 1:10,000, to normalize the sample loading. Horseradish peroxidase–conjugated anti-rabbit secondary antibody from Santa Cruz was used at 1:3,000 dilution. Antibody reaction was visualized using ECL Western blotting detection reagents (Amersham-Pharmacia, Uppsala, Sweden). The experiments were done in duplicate.

3.6. Statistical analysis

Statistical analyses were carried out using SPSS 17.0 for windows (SPSS Inc., Chicago, IL). Standard descriptive analysis was applied to describe all the variables. Differences between the groups of tumours were compared according to Mann Whitney U-test or Chi square test. P-values < 0.05 were considered statistically significant. Experimental values were expressed as mean±SEM.

4. RESULTS

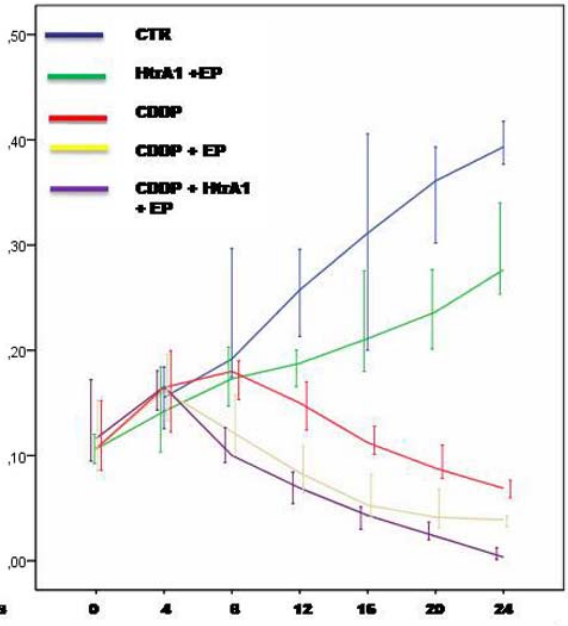
4.1. EP enhances *in vivo* uptake of HtrA1 expression vector in a mesothelioma xenograft

In order to analyze if EP of a HtrA1 expression vector in MM xenograft was able to increase the expression of HtrA1 in the tumors, we performed western blot analysis on proteins extracted from both control tumors and tumors electroporated with HtrA1. In order to exclude any effect eventually caused by EP alone, the control tumors were also electroporated. As shown in panel A of Figure 1, the protein level of HtrA1 was significantly higher in the tumors electroporated with the HtrA1 plasmid respect to the control tumors. As a further demonstration of this phenomenon, we performed immunohistochemical staining for HtrA1 on the same group of tumors. As shown in Panel B of figure 1, the expression of HtrA1 was clearly detected in the cytoplasm of the neoplastic cells and was significantly higher in the tumors electroporated with HtrA1 plasmids respect to the control tumors.

4.2. EP of HtrA1 slows tumor growth *in vivo* and strengthen CDDP cytotoxicity in a mesothelioma xenograft

It has been shown in several scientific works that EP alone has no effect on tumor growth *in vivo*, therefore tumors treated with EP alone were considered as control (22). The treatment with HtrA1 by means of EP was able to clearly slow the growth of the tumors, reaching a significance at day 20 ($p<0.0001$). The treatment with CDDP was, indeed, effective in reducing tumor volume, as expected. Interestingly, the combined treatment with CDDP and EP was more effective than the treatment with CDDP alone, reaching a significant value at day 12 ($p<0.0001$). Finally, the combination of EP with CDDP and HtrA1 was the most effective treatment in reducing tumor volume, reaching a border line significant value respect to the treatment with CDDP and EP at day 24 ($P=0.006$). The absolute tumor measures for the four groups as well as the graphic with the tumor growth curves are reported in Figure 2.

Histopathology analysis on control samples evidenced tumour tissue with high mitotic index. Treatment with CDDP caused partial substitution of the tumour tissue by calcified and necrotic tissue. This phenomenon was significantly enhanced in the tumours treated with EP, CDDP and HtrA1 where it was registered almost complete destruction of the tumour tissue and its replacement by scar and necrotic tissue (Figure 3). More importantly, the combination of CDDP and EP resulted in higher degree of tissue retraction during the scarring process secondary to



CTR vs HtrA1+EP	0.919	0.950	0.230	0.029	0.061	<0.001	<0.001
HtrA1+EP vs CDDP	0.848	0.711	0.935	0.002	<0.001	<0.001	<0.001
HtrA1+EP vs CDDP+EP	0.903	0.971	0.004	<0.001	<0.001	<0.001	<0.001
CDDP+EP vs CDDP+HtrA1	0.951	0.906	0.320	0.049	0.073	0.027	0.006
CDDP vs CDDP+EP	0.750	0.631	0.009	<0.001	<0.001	<0.001	0.002

Figure 2. *In vivo* analysis of the tumor growth in implanted mice. The graphic in the upper portion of the figure shows the growth curves of the tumors treated only with electroporation (control), electroporation plus HtrA1, CDDP alone, electroporation plus CDDP or electroporation plus HtrA1 and CDDP; the tumor volumes are expressed in mm^3. Each point represents the mean ± SD of 7 tumors. The table in the lower portion of the figure indicates the statistical value of the different volumes in each group.

tumour regression, while the cohort receiving HtrA1 plasmid had a faster recovery and lesser fibrous retraction (data not shown).

5. DISCUSSION

There is not a consensus on the best therapeutic approach to MM and the prognosis for this neoplasm is still dismal, survival being limited to a median of 8 to 18 months after diagnosis (3). The role of surgery is questionable in many patients, although cohorts of selected patients evidenced long term control in a small number of patients (38,39). Radiation therapy is frequently confined to a palliative role, due to the widespread extension of the disease at the time of diagnosis and to the possible radiation-induced side effects (40-42). Chemotherapy studies on patients with MM used either single agents or in combination. Generally, single agent chemotherapy yield response rates lower than 20% and do not bear a clear survival advantage (43). Combination chemotherapy is also associated with a poor response rate (20%) however some combination of oxaliplatin with pemetrexed or gemcitabine led to higher rates of response, although the overall survival time remained short (median 9-18 months) (44).

New drugs are currently under evaluation for MM in several controlled trials (see: http://www.cancer.gov/clinicaltrials). Among the others, Rapirnase a protein synthesis inhibitor, EGFR inhibitors, antiangiogenic agents and tyrosine kinase inhibitors show promises (45,46). Moreover, several treatment induced side effects have been reported, including peritoneal adhesions as serious sequelae of surgery, that can cause significant morbidity and/or mortality due to pain, infertility, and bowel obstruction, catheter complications associated with intra-cavitary chemotherapy and chemotherapy/radiation therapy associated pneumonitis (47-49).

The definition of molecular mechanisms able to potentiate the effects of CDDP both in *in vitro* and *in vivo* MM models could represent the scientific rationale from which starting to define novel and more efficacious local and systemic therapies to add to the therapeutic schemes already adopted to improve palliation and possibly to increase the therapeutic index for these patients. The rationale of this work is essentially based on three different observations of our research group.

First, we have shown that expression of HtrA1 influences tumour response to chemotherapy by modulating chemotherapy-induced cytotoxicity. In fact, down-regulation of HtrA1 attenuated cisplatin and paclitaxel-induced cytotoxicity, while forced expression of HtrA1 enhanced cisplatin and paclitaxel-induced cytotoxicity.

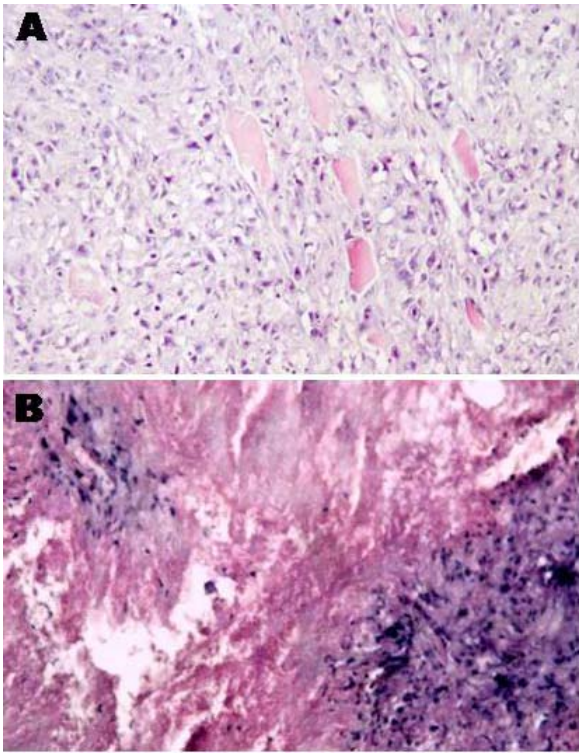


Figure 3. Histopathology analysis of the tumor xenografts at the end of the treatment. Panel A shows a control tumor treated with EP alone, while panel B shows a tumor treated with EP, CDDP and HtrA1: note the almost complete destruction of the tumour tissue and its replacement by scar (original magnification X20).

These findings uncover a novel pathway by which serine protease HtrA1 mediates paclitaxel- and cisplatin-induced cytotoxicity and suggest that loss of HtrA1 in cancers may contribute to *in vivo* chemoresistance (25).

Second, we have analyzed the possible involvement of HtrA1 in MM pathogenesis and progression. We have evaluated the immunohistochemical expression of HtrA1 in a well characterized cohort of 70 human MM. Indeed, we found a positive statistically significant relation between HtrA1 expression level and survival ($P < 0.0001$). The data obtained strongly indicate the utilization of HtrA1 expression as a prognostic parameter for MM and suggest this serine protease as a possible molecular target for the treatment of MM (32).

Third, we have investigated in the last years the possible application of EP for the therapy of cancer. Indeed, electrochemotherapy is a new approach to solid neoplasm that associates the administration of a chemotherapy agent to the application of square or biphasic electric pulses so to increase the uptake of drug by the cancer cells (39). EP is able to open transient and long-lasting pores within the cytoplasmic membrane, thus increasing the uptake of anticancer agents up to 700 folds. In particular, the solution of delivering the pulses as a train of biphasic waveforms adopted by our group has shown higher proficiency in inducing cross-membrane flow of molecules in targeted cells (40). The adoption of biphasic pulses has been extremely successful at disrupting the tumor membrane integrity in lieu of the particular arrangement of tumor cells, caused by the lack of size and shape homogeneity within cancers and by their frequent different orientation with respect to the field polarity, thanks to the sudden change of the electrons flow direction.

Considering that MM is currently an incurable disease for most patients, despite the adoption of multi-modality therapies, it is of paramount importance to develop novel strategies to palliate or extend the disease-free and overall survival of mesothelioma patients. In this experimental approach, based on platin compounds, we have combined the use of HtrA1 shown to potentiate the action of cisplatin, with EP. Indeed, we have demonstrated that EP was effective in favoring the expression of exogenous HtrA1 in MM cells and that this over-expression was able to slow the growth *in vivo* of MM xenografts respect to the control, coherently with the tumor suppressor-like activity of HtrA1 (33). Nevertheless, CDDP coupled with ECT yielded a better result in term of tumor reduction respect to the activity of CDDP alone. Finally, the combination of CDDP, HtrA1 and EP was even more efficacious respect to the activity of CDDP plus EP in reducing tumor volume. Nevertheless, we observed a decreased production of fibrous tissue, this resulting in decreased tissue retraction in rodents receiving the triple combination. If confirmed by further trial in humans, this approach might extend the disease free interval in patients with MM, decreasing the incidence of post treatment complications. Furthermore, this multi-modality approach could be very effective in improving local tumor control after surgery. In fact, a substantial number of patients develop early local recurrence, even with radical resection combined with aggressive chemo- and radiotherapy. The identification of effective combination coupled with the development of novel equipments and electrodes will be instrumental in planning the translation of these results to humans as per correct laboratory-clinical interface. Nevertheless, survivors of surgical and radio-therapeutic management may suffer from sequelae of treatment ranging from pain and infection to partial loss of organ function. Such high morbidity and mortality are truly regrettable. Our approach as shown by several phase II trials performed in companion animals with high grade spontaneous neoplasm (41,42,43) led to prolonged local control with minimal rate of complication. Furthermore, EP can be performed adopting different drug schedules in patients experiencing recurrence without the well known radiation-associated complications (bleeding, extensive fibrosis, pneumonitis and difficult wound healing). Finally, the low cost of the equipment, the ease of administration of anti-tumor drugs and genes, the absence of systemic toxicities, could facilitate the diffusion of this therapy.

6. ACKNOWLEDGMENTS

This work was supported by grants from Futura-onlus and Second University of Naples to AB and by ACC and Ministry of Health grants to EPS.

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Key Words: Electrochemotherapy, HtrA1, CDDP, Biphasic Pulses, Mesothelioma, Xenograft

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