IL NUOVO CIMENTO **43 C** (2020) 135
DOI 10.1393/ncc/i2020-20135-y

Colloquia: SIRR 2020

Matrix metalloproteases regulation in colorectal adenocarcinoma cells exposed to X-rays

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received 25 January 2021

Summary. — Matrix metalloproteases (MMPs) are degradation enzymes that allow cells to migrate through the extracellular matrix. MMPs generate new binding sites for tumour cell receptors that stimulate migration and invasion of normal tissues, leading to metastasis. In our Radiation Biophysics and Radiobiology Laboratory we have started an extensive experimental characterization of the response to X-rays (up to 10 Gy) of Caco-2 cells: this cell line is derived from human colorectal adenocarcinoma, usually adopted as an intestinal barrier model and recently characterized as radio-resistant. Colorectal cancer is among the three top cancer types for incidence and the second for mortality, usually treated with surgery, chemotherapy and radiotherapy. We performed gelatine zymography analysis, to evaluate MMP-2 and MMP-9 activity, which has been little investigated as a function of radiation dose and time after exposure. We here report results on the dose-dependent inhibition of these MMPs, also showing that measurements of MMPs activation can be severely affected by the choice of the experimental protocol, and particularly by the temporal sequence of radiation treatment and cell seeding.

1. – Introduction

Matrix metalloproteases (MMPs) are a group of proteins synthetized in an inactive form (zymogens) that require calcium-zinc ions for their enzymatic activity [1]. To activate MMPs, the pro-domain in the zymogen structure (pro-MMPs) has to be removed, so that the catalytic domain is free to bind and digest the extracellular matrix (ECM) [2]. This activation process can be started by different events, *e.g.*, the direct cleavage of endopeptidases or chemical modification [3]. The activation of pro-MMP is a gradual process that occurs in the pericellular space. The first step includes a conformation change of the pro-peptide; subsequently, the pro-domain is removed by intra- or inter-molecular interactions [2]. *In vitro* experiments demonstrated that the incubation of recombinant pro-MMPs with thiol-modifying chemical agents, *e.g.*, SDS, or reactive oxygen species

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(ROS), induces the activation of several MMPs. *In vivo*, ROS are produced by enzymes present in neutrophils and macrophages during tissue injury including radiation exposure, but it is still not demonstrated that ROS can directly induce the activation/inhibition of MMPs [4]. MMPs can be classified in different categories, due to their substrate specificity, *e.g.*, collagenases, gelatinases, stromelysins, matrilysins [1]. Over many years, MMPs have been considered potential diagnostic and prognostic biomarkers for different types and stages of cancers [5, 6]. These enzymes are involved not only in the ECM remodelling that leads cancer cells to migration, invasion, metastasis and angiogenesis (four hallmarks of cancer), but also in cell proliferation, apoptosis and regulation of cell cycle [7]. MMPs play a key role in morphological modifications, in particular regulating the apico-basal polarity, *e.g.*, the de-differentiated phenotype acquired through an epithelial to mesenchymal transition (EMT), as observed in some epithelial non-cancer stem cells (CSCs) [8]. MMPs are also involved in activation and release of different chemokines, cytokines and growth factors in response to inflammation, as demonstrated with a co-culture setup between Caco-2 (colorectal adenocarcinoma cell line) cells and peripheral blood mononucleated cells (PBMCs) [9]. All these considerations lead to the involvement of MMPs in the complex response activated by cells exposed to ionizing radiation (IR) [10]. In this work, using an *in vitro* model of colorectal adenocarcinoma cells (Caco-2) exposed to X-rays, we focus on how MMPs activation depends on radiation dose and time after exposure, as well as on how it is affected by the choice of the experimental protocol.

2. – Materials and methods

2˙1. *Cell culture and irradiation protocols*. – Caco-2 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM (Gibco)) supplemented with 10% fetal bovine serum (FBS (Life Technologies-Gibco)), 2 mM L-glutamine (Life Technologies-Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies-Gibco) at 37 °C in a humified atmosphere with 5% CO_2. Irradiations were performed at the radiotherapy department of *Istituto di Ricovero e Cura a Carattere Scientifico* (IRCCS) S. Maugeri (Pavia, Italy) with a linear accelerator routinely used for radiotherapy treatment, as previously described [11].

2˙2. *Experimental procedures*. – Experiments were performed using two different approaches; in the "Seed + Treat" method cells were seeded in T25 flask and, after 48 h from seeding, cells were exposed to X-rays. In the "Treat + Seed" method, cells were exposed to X-rays and then plated in 60×10 mm^2 Petri dishes. For both approaches, cells were exposed to 0 Gy (Sham), 2 Gy, 5 Gy and 10 Gy, and 6, 24 and 48 h after exposure supernatants were collected for MMP measurements.

2˙3. *Gelatine zymography*. – Measurements of Matrix Metalloproteases (MMP-9 and MMP-2) in the culture medium were performed following the experimental procedure already published in [12], with minor changes. Conditioned media were collected, centrifuged at 4600g (Thermo Scientific CL31R) and supernatants mixed in Sample Buffer 2X (0.5 M Tris-HCl pH 6.8, 20% glycerol, 10% SDS, 0.1% Bromophenol blue), ratio 1:1, and stored at -80 °C. 20 μl of each sample were loaded on a 10% polyacrylamide gel containing 1 mg/ml Bovine Type B Gelatine (Sigma-Aldrich). Gels were stained with Coomassie Blue R-250 (0.5% w/v) and subsequently de-stained and acquired with Image Gel Analyzer (Bio-Rad).

2˙4. *Quantification and statistical analysis*. – The intensity of white bands, corresponding to the gelatinolytic activity of MMP-9 and MMP-2, was quantified by ImageJ [13], and expressed in relative percentage to that of Sham samples (un-irradiated controls) at the same time point. Errors are expressed as standard deviations of the mean of at least three independent experiments. When shown, statistical significance was calculated by two-tailed Student's t-test.

3. – Results and discussion

Gelatine zymography is an electrophoresis technique with the addition of bovine gelatine type B in an acrylamide gel, that acts as a substrate for MMP-9 and MMP-2. These two MMPs are also considered gelatinases, due to their gelatinolytic activity. The run occurs in denaturing conditions, in order to allow their electrophoretic migration, and in non-reducing conditions, in order to avoid the reduction of cysteine residues that would compromise the gelatinolytic activity. The renaturation of the MMPs occurs with the removal of the SDS contained in the running buffer, by a Triton-X 100 buffer. The white bands (fig. 1) represent where the MMPs digested the gelatine. The intensity of white bands, proportional to MMP activity, is quantified by ImageJ and expressed as relative percentages with respect to the Sham condition at the same time point. It is important to state that this technique has to be considered as semi-quantitative, and limitations exist for the quantification of results.

Experiments were carried out in parallel with two different experimental protocols to evaluate MMPs activation following X-ray exposure of Caco-2 cells. Specific aim of this work was to evaluate to what extent such results are affected by the protocol itself, in particular by the use of trypsin (an enzyme commonly used to detach cells and re-seed them). The correct choice of the methodological approach affects data interpretation (fig. 2): indeed, in the "Seed + Treat" method we observed an inhibition of MMP-2 activity as radiation dose increases, particularly at the highest 10 Gy dose. At this high dose cell death mechanisms are also activated [14], presumably including apoptosis. Interestingly, data from Chetty *et al.* [10] on a lung cancer cell line indicate that inhibition of MMP-2 is at the basis of an enhancement of radio-sensitivity. This seems to be coherent with a specific role of MMP-2 in the radiation response. Comparing results obtained with the two different approaches, we can hypothesize that trypsin impaired the MMPs inhibition in the "Treat + Seed" protocol; as similarly observed by Löffek *et al.* [4], trypsin can cleave the pro-peptide inducing the MMPs activation, thus prevailing on the radiation-induced inhibition of MMPs. This suggests that, once the correct protocol is chosen, a dose-dependent inhibition of MMPs activation can be measured. Additional

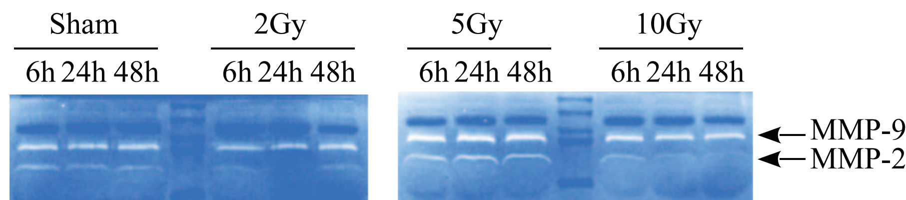

Fig. 1. – Representative images of gelatine zymography, performed for samples of the "Treat + Seed" method.

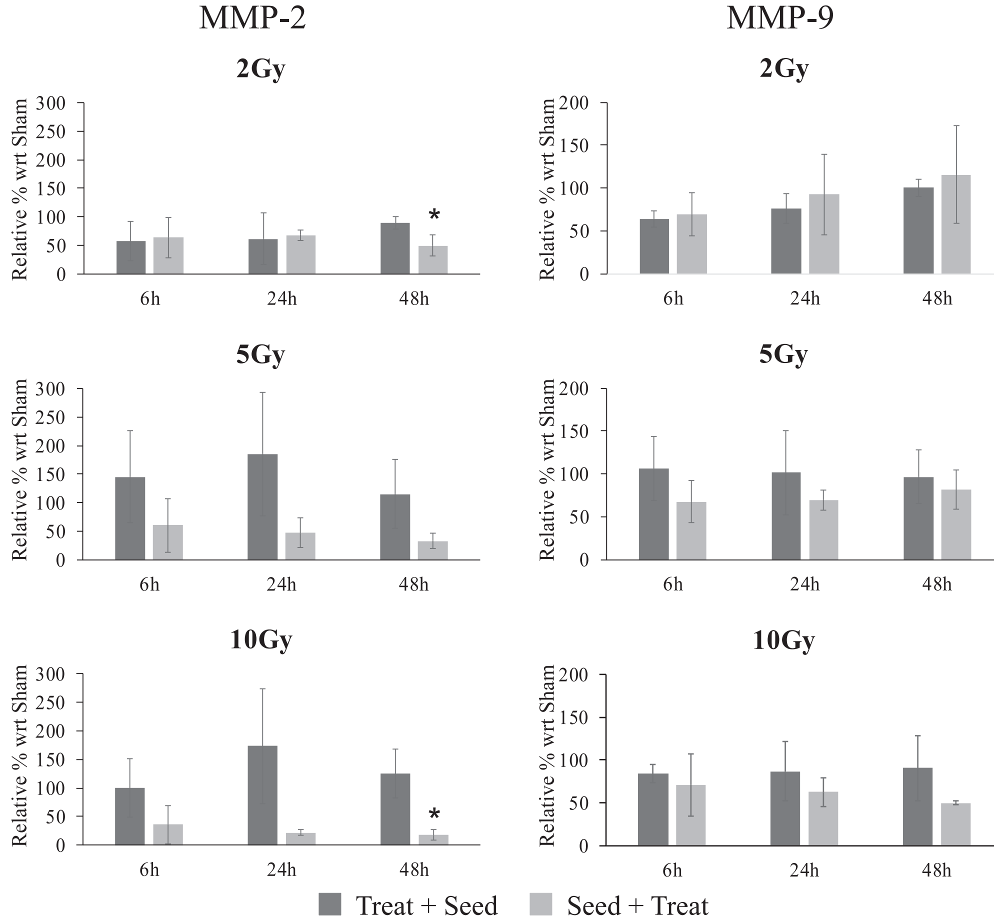

Fig. 2. – Quantification of MMP-9 and MMP-2 by ImageJ at different radiation doses and times after exposure expressed as relative percentage with respect to Sham (0 Gy) at each time point. Histograms represent the comparison between the "Seed + Treat" and the "Treat + Seed" protocols. Errors are expressed as standard deviations of the mean of at least three independent experiments and statistical significance was calculated by two-tailed Student's t-test, ${}^{\star}p < 0.05$.

endpoints will be investigated to obtain a more complete overview of the complex response of Caco-2 cells to radiation.

4. – Conclusions

Ionizing radiation (IR) is commonly used as clinical treatment for colorectal cancer, the third cancer ranked for incidence worldwide [15]. The most common treatment for colorectal cancer is surgery; however, radiotherapy and chemotherapy play important roles as adjuvant or neo-adjuvant therapies, respectively to allow conservative resection or to reduce the probability of secondary local tumours [16]. IR produces DNA damage, inducing the activation of a signalling cascade mechanism that, among other effects, results in the phosphorylation of the Chk2 protein, responsible for regulating the transition

of cells from the G2 to M phase. The blocking of this transition, with consequent DNA repair, is one of the main strategies that cells can activate to acquire radio-resistance, and for this reason, as far as cancer cells are concerned, checkpoints are considered good therapeutic targets [17]; cells unable to repair DNA damage and to progress through the cell cycle can activate cell death pathways [18]. The correct regulation of Chk2 is also performed by MMP-2, whose activity is reduced in case of radiation exposure [10]. Results presented in this work suggest a dose-dependent inhibition of the activation of MMPs. However, we here show that great care is needed in the choice of the experimental protocol to perform these measurements: in particular, the use of trypsin after radiation treatment activates MMPs, prevailing on their radiation-induced inhibition. Results on dose-dependent MMPs activation will be further integrated in a wider framework, studying their correlation to cell death and cell cycle perturbation, in the complex interplay leading to sensitivity/resistance of cancer cells.

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