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Changes in the quantity of cathepsin D in irradiated human cells following treatment with hyperthermia and interferon α

Ana Ferle-Vidović,[1] Marija Kaštelan,[1] Danilo Petrović,[1] Janez Škrk,[2] Ivan Vrhovec[2]

[1] Ruđer Bošković Institute, Zagreb Croatia, [2] Institute of Oncology, Ljubljana, Slovenia

In the present work, the changes in quantity of cathepsin D, an aspartic proteinase, in proliferating human nonmalignant (HEF) and malignant (HEp2) cells after combined treatment by gamma irradiation, hyperthermia and by interferon α-2b (IFNα) were followed. Correlation between the antiproliferative effect of these combined agents and changes in the concentrations of this cathepsin D were expected. Evidently, the treatment of cells in culture by IFNα, combined with irradiation and elevated temperature, produces an increased quantity of cathepsin D. In nonmalignant HEF cells these effects are more expressed than in malignant HEp2 cells.

Key words: cathepsin D; cell, cultured-radiation effects; hyperthermia, induced; interferon-alfa-2B

Introduction

Intracelular proteinases participate in the vital cellular processes such as growth and multiplication, response to DNA damage and radiation response.[1, 2, 3, 4] Recent studies suggest that the aspartic proteinase cathepsin D, may also be implicated in the process of tumor invasion and metastasis,[5] Several *in vitro* observations showed that this proteinase may facilitate the spread of neoplastic cells through different mechanisms releated to its proteolytic activity, by acting at different levels of the metastatic cascade. Cathepsin D was also shown to be able to degrade *in vitro* the extracellular matrix, and to activate latent precursor forms of other proteinases involved in the invasive steps of the metastatic process.

Interferon α-2b (IFNα) initially recognized for its antiviral effects, has also been shown to have antiproliferative, immunoregulatory and antitumor activities.[6] There is some experimental evidence supporting the concept that modest levels of hyperthermia might be beneficial to the action of interferons. This concept is supported by *in vivo* experiments showing that modest levels of hyperthermia enhanced the action of interferon.[7] *In vitro* it was found that hyperthermia acted synergistically by enhancing the proliferative effects of IFNα.[8] There is definitive experimental evidence *in vivo* for synergistic effect of this combined treatment.[9] Similar synergistic effects were also observed, when interferon was applied in combination with irradiation.[10]

Correspondence to: Ana Ferle Vidović, Ph. D., Ruđer Bošković Institute, Bijenička cesta 54, 41000 Zagreb, Croatia

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Such results may have clinical importance, because they suggest that hyperthermia could be used in combination with IFNα to provide a synergistically enhanced antitumor action.[9, 10] Therefore, it is possible that the combination of hyperthermia and IFNα therapy may have clinical application in cases when technically feasilbe. Such combined treatment can, however, induce an increase in proteolitic enzymes in cells, which might enhance metastasing of the treated tumor.

In this work we determined the changes in the quantity of the aspartic intracellular proteinase – cathepsin D in irradiated human nonmalignant (HEF) and in malignant (HEp2) cells after combined treatment with hyperthermia and/or IFNα. Correlation between the antiproliferative effect of these combined agents and the changes in concentrations of this cathepsin was found.

Materials and methods

Cell cultures and experimental procedure

Human embrional fibroblasts (HEF) and human laryngeal carcinoma (HEp2) cells, were cultured as monolayers in Eagle's minimal essential medium, supplemented with 10 % foetal calf serum. Cell cultures were prepared by plating 10^5 of 10^6 cells per Petri dish of 10 cm in diameter (3 dishes per experimental point) and after two days of growth, cells were irradiated, treated by IFNα and by hyperthermia in the following combinations: irradiation only, IFNα only, irradiation plus IFNα, irradiation plus IFNα plus hyperthermia.

Following the mentioned treatment, cell cultures (10^6 per dish) were kept at 37°C and samples taken after one hour were stored at –20°C until proteinase quantity assay. The number of proliferating cells (10^5 per dish) following irradiation and combined treatment were counted 24 and 96 hours after treatmen.

Interferon

Recombinant interferon α-2B (INTRON-A, Schering-Plough-Baltimore-USA) was added to the growth medium to reach final concentration of 1×10^4IU/ml. Cells were incubated in the IFNα-containing medium at 37°C for 1 hour and then incubated until additional treatment or harvesting.

Hyperthermia

Heat treatment was conducted by submerging the Petri dishes in a water bath at 44°C for 20 min. HEF and HEp2 cells were exposed simultaneoulsy to IFNα and to hyperthermia.

Irradiation

For gamma irradiation, a Gamma Cell 220 (Atomic Energy of Canada, αtd) unit was used. The dose rate was 4,13 Gy/min, with the total dose 15 Gy/sample for cathepsin D, or 5 Gy for the growth inhibing effect.

Cathepsin D concentration

Following treatment, cell cultures were incubated in the growth medium at 37°C. Samples were taken after different time intervals, placed on ice and washed three times with cold phosphate buffered saline. The cells were harvested by a rubber policeman, concentrated by centrifugation (10 min at 1000 rpm), lysed in distilled water and frozen at –20°C until assay.

The concentrations of cathepsin D were determined using specific enzyme immunometric assay (ELSA-CATH-D kit, CIS Bio International, Solid phase two-site immunoradiometric assay), for the quantitative determination of total cathepsin D in cytosol.

Results

The combined effects of IFNα, hyperthermia and irradiation on cell proliferation, expressed as the number of growing HEF cell population, are shown in Table 1. The antiproliferative effects were expressed as percentage of cell numbers in control samples. While irradiation or IFNα alone, moderately inhibited cell growth, the treatments, by IFNα plus irradia-

tion, and in particular, when IFNα was combined with irradiation and hyperthermia, the antiproliferative effect was markedly enhanced.

Table 1. Cell numbers (x10^5 HEF cells) after combined treatment with irradiation and/or interferon α and/or hyperthermia.

Group of treatment	Incubation time after treatment		Percent of control	
	24^h	96^h	24^h	96^h
Controls	10,9 ± 2,6	20,4 ± 3,1	–	–
Irradiation	5,0 ± 0,9	7,5 ± 1,1	46	37
Interferon α	6,6 ± 0,2	8,7 ± 1,7	61	43
Irradiaton + Interferon α	3,7 ± 0,1	4,0 ± 0,4	34	20
Irradiation + Interferon α + Hyperthermia	1,8±0,1	1,9 ± 0,2	16	9

The combined effects of IFNα, hyperthermia and irradiation on cell proliferation, expressed as the numbers of growing HEp2 cell population, are shown in Table 2. The antiproliferative effects were expressed as percentage of cell numbers in control samples. While irradiation or IFNα alone, moderately inhibited HEp2 cell growth, the treatmens by IFNα plus irradiation, and in particular when IFNα was combined with irradiation and hyperthermia, the antiproliferative effect was markedly enhanced.

Changes in the concentrations of cathepsin D measured in irradiated, proliferating human nonmalignant (HEF) and human malignant (HEp2) cell lines following combined treatment by interferon α and by hyperthermia are shown in Table 3.

Table 2. Cell numbers (x10^5 HEp2 cells) after combined treatment with irradiation and/or interferon α and/or hyperthermia.

Group of treatment	Incubation time after treatment		Percent of control	
	24^h	96^h	24^h	96^h
Controls	16,4 ± 1,4	41,0 ± 4,2	–	–
Irradiation	12,0 ± 0,9	18,6 ± 1,6	73	45
Interferon α	11,0 ± 2,1	21,8 ± 4,0	67	53
Irradiation + Interferon α	8,0 ± 1,1	10,0 ± 0,7	48	24
Irradiation + Interferon α + Hyperthermia	3,6 ± 0,8	7,7 ± 0,7	22	18

The changes in the concentrations of cathepsin D were dependent on the agent used. Gamma iradiation alone revealed little change in the concentration of cathepsin D in the malignant cell line (1.1), whereas the same changes were more evident in the nonmalignant cells (1.3). Interferon α increased the levels of cathepsin D in both cell strains. These effects are more expressed in malignant cells (1.5), than in nonmalignant cells (1.2). Interferon α plus irradiation increased the concentration of cathepsin D significantly more than in the former two cases, when the agents were applied separately. The increased concentrations of the enzyme were similar in malignant (1.6) as in nonmalignant (1.8) cells. Combined application of all three agents together was most effective in increasing the amounts of cathepsin tested. Most evident effects were achieved in both cell lines after combined treatment with all three agents and again, with more expressed effects

Table 3. Changes in the quantity of Cathepsin D in irradiated HEF and HEp2 cells following combined treatment.

Group of treatment	HEF cells		HEp2 cells	
	Cathepsin D ng/mg proteins	T/C*	Cathepsin D ng/mg proteins	T/C*
Controls	105 ± 11	–	562 ± 14	–
Irradiation	138 ± 7	1,3	604 ± 12	1,1
Interferon α	127 ± 9	1,2	875 ± 22	1,5
Irradiation + Interferon α	198 ± 26	1,8	856 ± 31	1,6
Irradiation + Interferon α + Hyperthermia	332 ± 16	3,1	1407 ± 53	2,5

* T/C = Treated/Control

on the nonmalignant HEF cell line (3.1), the in the malignant HEp2 cells (2.5).

Discussion

Cathepsin D, as mentioned before, plays an important role in tumor invasion and metastasis. Correlation between elevated levels of the enzyme in tumor cells and their ability to metastasise were found.[5] On the other hand in our previous experiments we found that agents used in tumor therapy can influence the concentrations of various intracellular proteolytic enzymes, either by increasing or decreasing their concentrations[11] or activities.[12] This raises the question, particularly when cathepsin D is concerned, whether a particular tumor treatment could perhaps, apart of its cell killing potential, have some unwanted effects due to possibly elevated levels of cathepsin D.

Our results show that such agents (irradiation, interferon α and heath), when given in amounts that evidently produce cell growth inhibition, can significantly increase the intracellular concentrations of cathepsin D, and therefore could consequently enhance the potential of the tumor cells to infiltrate the neighbouring tissues or to metastasise. If this occurs in a tumor bearing organism, this should also be kept in mind, when predicting the outcome of a particular tumor therapy. This may be even more important at combined modality therapy regimens.

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