Chirurgia (2014) 109: 806-811
No. 6, November - December

Involvement of Reactive Oxygen Species in the Mechanisms Associated with Cervical Cancer Specific Treatment

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Rezumat

Implicarea speciilor reactive ale oxigenului în mecanismele de semnalizare specifice asociate tratamentului specific cancerului de col uterin

Cancerul colului uterin reprezintă o adevărată problemă de sănătate publică în România. Tratamentele instituite sunt complexe, iar mecanismele biochimice care le însoțesc nu sunt încă elucidate pe deplin. Astfel, radioterapia, care este inductoare de specii reactive ale oxigenului poate să aibă eșecuri în țesuturile hipoxice, țesuturi greu de identificat, deoarece producerea acestor specii citotoxice are loc în concentrații mici. Drept urmare, scopul lucrării este de a identifica producerea și rolul speciilor reactive de oxigen, precum și modul de activare al apărării antioxidante endogene la pacientele cu cancer al colului uterin tratate în Institutul Oncologic București. Pentru aceasta s-au determinat parametrii biochimici de stres oxidativ la un lot de 30 paciente cu această localizare tumorală, înaintea intervenției chirurgicale. Rezultatele pe care le-am obținut au arătat că la aceste paciente are loc o producere de specii reactive de oxigen, care atacă ca și țintă primară lipidele, provocând o peroxidare a acestora. Extensia degradării oxidative a proteinelor are loc la o valoare mult mai mică, iar activarea sistemelor endogene de apărare antioxidantă se produce la o rată mult mai scăzută. În concluzie, considerăm că atunci când producerea metaboliților activi ai oxigenului se face în concentrații mici, asociate cu hipoxia, semnalele transmise sunt spre modificarea unui fenotip din condiții anaerobe spre unul care să activeze neovascularizația, inițierea angiogenezei, creșterea și proliferarea de noi celule. În momentul în care s-a depășit această fază, se instalează un stres oxidativ care poate fi distructiv pentru biomoleculele esențiale vieții, dar și util tratamentelor ulterioare, cum ar fi radioterapia.

Cuvinte cheie: cancer col uterin, rezistența la tratament, mecanisme de semnalizare a hipoxiei, specii radicalice ale oxigenului

Abstract

Cervical cancer represents a genuine health issue in Romania. The courses of treatment applied are complex, and the accompanying biochemical mechanisms are yet to be fully understood. Thus, radiotherapy, which induces reactive oxygen species, can lead to failure of treatment in hypoxic tissues, tissues which are difficult to identify due to the small quantity in which these cytotoxic species are produced. As a result, the aim of this paper is to identify the production and role of reactive oxygen species, as well as the manner of activation of endogenous antioxidant defense mechanisms in cervical cancer patients admitted to the Oncologic Institute of Bucharest. To this purpose the biochemical parameters of oxidative stress were identified in 30 patients with cervical tumour localization, prior to surgery. The results obtained have showed that a production of reactive oxygen species is identifiable in these patients, having lipids as a primary target and leading to their peroxidation. The extension of protein oxidative degradation takes place at a much lower

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value, as well as the activation of endogenous antioxidant defence systems, comparing to our expectations. To conclude, we consider that when the production of active oxygen metabolites takes place in small concentrations, associated with hypoxia, the signals transmitted are towards modifying the phenotype under anaerobic conditions into one activating neovascularization, angiogenesis initiation, new cell growth and proliferation. The moment that this phase is overcome a new oxidative stress is installed, one potentially destructive for biomolecules essential to life, but also useful for further treatment, such as radiotherapy.

Key words: cervical cancer, treatment resistance, hypoxia signalling mechanisms, reactive oxygen species

Introduction

Cervical cancer is found in fourth place in terms of incidence among cancers developed by women, with approximately 530,000 new cases worldwide in 2012 (1). Cervical cancer represents a genuine public health issue in Romania. It is the second leading cause of mortality through cancer (the first is breast cancer), but is assigned first rank in the 25-44 years age group. Although there are means of early identification (screening tests) and diagnosis, cervical cancer presents a series of problems related to treatment. Unfortunately, incipient cervical cancer is frequently asymptomatic. It is worth underlining that approx. 33% of stage III or stage IV patients describe the first occurrence of symptoms only 3 month prior to diagnosis. Hence the importance of regular screening by means of Babeş-Papanicolau cytological test. (2)

Treatment in the case of these types of neoplasia consists of the surgical intervention, followed by radio- or chemotherapy. Modern anaesthetic and surgical techniques allow the performing of the surgical operation in all safety, associating an operative mortality of approximately 0.6%. (3)

Radiotherapy can also be administered as primary therapy, but it can be used for patients initially treated by surgery with unfavourable prognostic factors as well.

Irradiation consists of an association between external and internal radiotherapy. Postoperative radiotherapy lowers the risks of pelvic recurrence in patients treated by radical hysterectomy with unfavourable prognostic factors (lymph node, parametrium invasion). (4)

A factor which plays an important role in the determination of tumour resistance to radiotherapy is hypoxia. Radiosensitivity decreases fast when the oxygen partial pressure (pO_2) is under 25-30 mmHg. Several theories have been elaborated in attempt to explain this fact. One of these involves intratumoral control of molecular oxygen, a potent radiosensitizer involved in the mediation of DNA degeneration. Oxygen presence may increase DNA alterations through production of free oxygen-derived hydroxyl radicals, which initially appear after interaction between radiation and intracellular water. On the other hand, oxygen presence can stabilize highly reactive hydroxyl radicals, producing alterations in DNA, and weakening thus cellular ability to fix altered DNA chains. Due to the effect of decreasing intracellular oxygen under hypoxic conditions the ionising radiation dosage of a single cytotoxic fraction capable of destroying a certain number of cells is approximately 2-3 times higher under hypoxic conditions than under normal ones. In other words, radiotherapy is 2-3 times less efficient for cells under hypoxic conditions than for those under normal oxygenation conditions. (5,6)

Cumulative proof indicate that progressive tumour development is dependent on the process of angiogenesis. Most tumours in humans persist in situ for long periods of time (months up to years), in a state of avascular rest. During this phase the tumour can consist of several millions of cells. When a subgroup of tumour cells shifts to an angiogenetic phenotype, by modifying the local balance between positive and negative angiogenesis regulation factors the tumour begins to grow rapidly, becoming clinically detectable. (7,8,9)

Oxidative stress can activate numerous intracellular signalling pathways mediated by ROS (reactive oxygen species), by modulating the activity of various enzymes and critical transcription factors. (10,11) In one scenario, the transcription factors activated in response to a ROS increase or to oxidative deterioration cross the cytoplasm towards the cell nucleus and bind to promoting regions of specific particular genes. (12) As a result, these pathways activated by oxidative stress can have a major impact on genetic expression, which will ultimately affect the fate of a cell (for example apoptosis or proliferation, cytokine production). The ratio between ROS production and the activity of cellular antioxidant endogenous defence systems can determine the activity of the signalling pathways related to oxidative stress and, implicitly, the fate of the cell, more precisely whether a cell exposed to a ROS increase is destined to survival or apoptosis. (13,14)

Aim

Bearing all of this in mind, the present paper is aimed at identifying the oxidative status in patients with cervical cancer prior to the surgical intervention, in order to determine the biochemical reactions potentially influencing the future evolution of the systemically monitored cellular phenotype. We plan on succeeding this by monitoring oxidative stress parameters, as indicators of disease evolution monitoring and treatment efficiency.

Material and Methods

Approval to conduct this study was obtained from the Ethics Committee of the Bucharest Institute of Oncology. We investigated 30 patients with stage II cervical cancer prior to surgical intervention and with no radiotherapy administered. After providing informed written consent, the patients' blood samples were drawn via venous puncture, prior to intervention.

Physiological serum was isolated by centrifugation, allowing for the following determinations:

- Lipid peroxides were evaluated by measuring the final concentration of malondialdehyde (MDA). The method is spectrophotometric and is based on the production of a coloured adduct (MDA-TBA2) with a maximum of absorption at 532nm depending on concentration. (15)
- The oxidative activity of ceruloplasmin can be measured through several methods. It is a pale blue protein with oxidase activity over polyamines, polyphenols and inorganic ferrous ions (Fe^{2+}) and a maximum affinity for them. The catalytic oxidation of Fe^{2+} or of complexes containing Fe^{2+} is known as ferroxidase activity. We employed the Ravin method by p-phenylene diamina reaction in an acetic acid – acetate tampon. It is, also, a spectrophotometric method based on the Lambert-Beer law, the intensity of the colour developed at 540 nm being directly proportional to the concentration of the measured compound. (16)
- SH thiol-albumin groups were determined by reaction to acid 5,5`-dithio-bis (2-nitrobenzoic) reactive Ellman, noted DTNB, which reaches as a result of various oxidations a maximum intensity at 412 nm, in accordance with the concentration of the newly formed SH groups (17).
- Total antioxidants were biochemically measured through a method that uses the serum's ability to reduce iron. At low pH, the Felll – tripiridyl-s- triazine (Felll –TPTZ) complex is reduced to ferrous form, with the generation of a new complex which develops an intense measurable blue hue, with a maximum absorption level at 593 nm. Any reaction with positive redox potential under the mentioned conditions can lead to the reduction of the Felll –TPTZ complex. Using a Fe excess, which is the limiting factor of the Felll –TPTZ complex and of colour development, we obtain the reducing ability of the sample. (18)

In order to perform these determinations the solutions were prepared using analytically pure compounds, from Merck and Sigma, used for clinical trials; the water used was genetically pure, as a result of filtering with Millipore (Milli-Q-Biocel) devices, and all spectrophotometric readings were performed on a Specord 210 (AnalyticJena) Spectrophotometer.

Results

Monitoring Lipid Peroxidation

In performing our determinations we started from the assumption that reactive species of oxygen produced in small concentrations play a part in signalling mechanisms that can change the cellular phenotype, more precisely shifting from a normal one to a phenotype in which angiogenesis and neovascularization mechanisms prevail. The latter mechanism leads to proliferation and cellular growth. On the other hand, excess production of reactive oxygen species becomes destructive and induces cellular lysis, which might be beneficial at tumour level. As a consequence, we determined in a first step the level of lipid peroxides within the study group. Individual result are presented in the following diagram (*Fig. 1*).

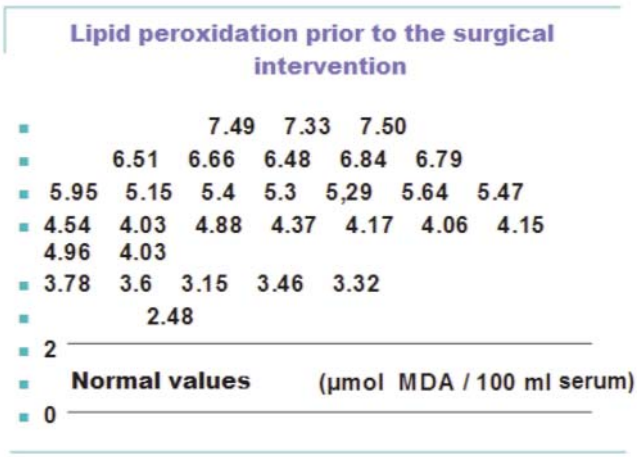

Figure 1. *Lipid peroxides values distribution*

Determining the oxidative activity of ceruloplasmin

Ceruloplasmin is an acute phase protein with intermediate amplitude response compared to other acute phase proteins, whose level is doubled or tripled in inflammation, pregnancy, traumatic lesions and surgical interventions. Ceruloplasmin, the transporting protein of copper, with a well-known ferroxidase activity, takes part in oxidation-reduction reactions which involve iron ions. Its known ferroxidase action, also determined in our study, suggest the existence of an oxidative stress as well, due to excessive generation of reactive oxygen species. The values recorded in cervical cancer patients prior to surgical intervention are presented in *Fig. 2*.

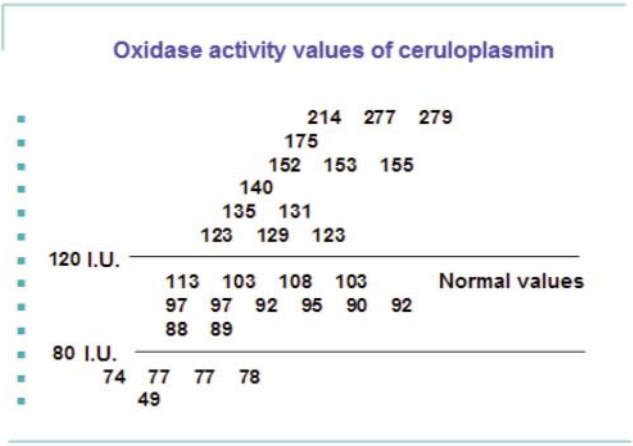

Figure 2. *Level of oxidase activity of cerulopasmin in patients with cervical cancer*

When interpreting the obtained results one can notice that 40% of the investigated patients present normal values of the studied parameter, 16% present with values under the normal threshold, and 44% show increased values up to twice the maximum normal level accepted in the literature for this technique.

In order to demonstrate this statement we have subsequently measured protein thiols production as a result of protein oxidative degradation; the result can be seen in *Fig. 3*. These data show that only 30% of the investigated patients present a value of protein thiols over the normal limit, resulted from oxidative degradation of circulating proteins, 23% present normal values of this parameter and a relatively high 47% percentage is represented by patients with values under the normal threshold.

We wished to demonstrate the presence of a system which develops oxidative stress by determining the total antioxidant status among the same study group. The values obtained are presented below (*Fig. 4*). The highest percentage, 64%, is attributed to patients with normal values of the parameter; while increased values were determined in only 33% of cases. These results suggest the possibility of endogenous protective antioxidant systems activation, but much slower than the production of reactive species.

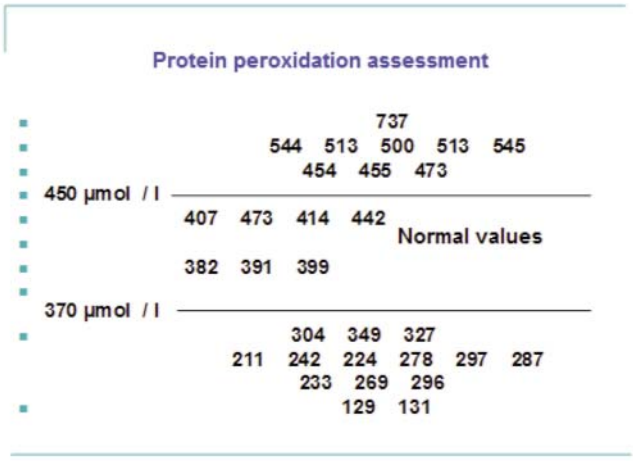

Figure 3. *Protein thiols production due to protein oxidation*

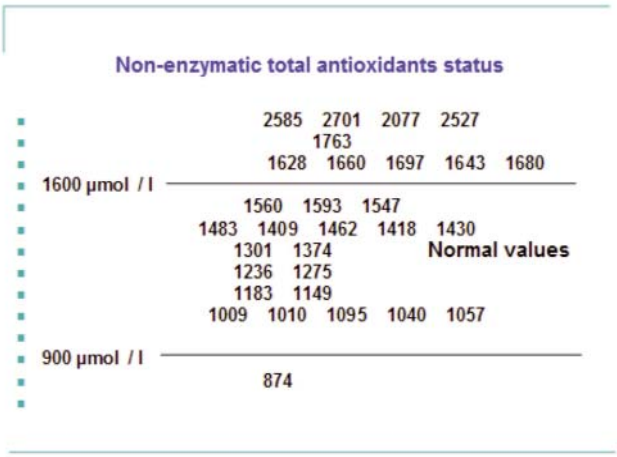

Figure 4. *Non-enzymatic total antioxidants serum values*

Discussions

Over the past 15 years the entire biomedical scientific world has endorsed a research campaign in the extremely controversial domain of free radicals and oxidative stress. Answers to a wide variety of questions regarding the biological role of reactive species are sought, free radical action mechanisms and their involvement in generating and/or aggravating human diseases being under study. More and more scientists are bringing arguments and proof supporting the involvement of reactive species in disease pathogenicity, but the aim of this research is to prevent development or disease progression influence by augmenting human cell population antioxidant defence. Another desired endpoint is a change in the mentality of those involved in health preservation and regain (doctors, biologists, hygienists, biochemists), who should be preoccupied with their patients having, among others, an adequate nutritional status, both before and after disease occurrence. In living organisms the presence of reactive oxygen/nitrogen species induces the rapid intervention of several antioxidant categories. Their importance depends on the type of reactive species occurring, on the way in which they were generated, on the place of occurrence and on the oxidative lesion target.

For example, when the body's fluids are exposed to the toxic activity of ozone or NO2, uric acid has an antioxidant protective role; on the other hand, urate does not protect plasmatic elements from the toxic activity of hypochlorous acid.

If the oxidative stress is the same, but different target lesions are measured, different results are obtained. For example, plasmatic exposure to cigarette smoke determined peroxidation of lipids and proteins; ascorbic acid protects lipids from the harming activity of cigarette smoke, but does not protect proteins as well.

ROS are capable of attacking different target molecules in vivo, including proteins, lipids and DNA.

The reasons for oxidative stress increase in tissue aggressions, including transitive metal ion discharge and of heme proteins with catalytic purpose, or mitochondrial lesions, are several.

ROS contribution to disease pathology can be significant or not, which is why it is very important to establish the degree to which these substances take part in lesion production in various afflictions. Up until not so long ago this was not possible due to lack of methods of detecting free radicals or their effects on tissues, with applicability in vivo.

Research conducted over the past 15 years has developed specific tests for detecting ROS through the method of "capture" and/or determining the resulting products of oxidative stress.

As can be observed in *Fig. 1*, the values of the final

oxidative lipid degradation product, malonaldehide, in the 30 patients investigated are between 2.48 – 7.50 μmols/100 ml serum, much higher than the normal 0-2 μmols/100 ml serum, which is also reported in the medical literature. These results entitle us to state that the increased level of the final product of lipid peroxidation is due to an intense oxidative reaction, consequence of the presence of reactive oxygen species generated primarily by oxygen metabolism. Compared to normal values, there is an increase in oxidative metabolism in these patients due to tumour presence.

One can state with certainty that lipids represent a target of the oxidative attack, and tumour presence induces reactive radical species. (19)

These increased values reflect the installing of an oxidation reactions, therefore reflecting the presence of anaerobic tissues which will not develop resistance during future treatment stages.

Thus, we are entitled to state that there is an overproduction of active oxygen metabolites participating in the ferroxidase reactions catalysed by ceruloplasmin, manifested in a smaller percentage of patients. Speculatively, we can say that the endogenous protective antioxidant mechanisms are also activated in these patients, due to proteins with such activity. (20)

Based on the data obtained from determining total thiols, we can specify that proteins are not the primary target of the oxidative attack. Sulphur proteins which are degraded to thiol metabolites are proteins with antioxidant activity. These are produced in excess by the tumour tissue in order to conceal the oxidative stress. Once again it is confirmed that the tumour tissue presents an oxidative metabolism different to normal tissues. As a response to the oxidative stress installed the endogenous protective systems are also activated. (21)

The results obtained have proved to us that reactive oxygen species production occurs in these patients, targeting lipids primarily, determining a cascade chain reaction, with excessive production of reactive metabolites, which can shortly migrate away from the generation site and attack other biomolecules essential to life (22). We have also observed the extension of protein oxidative degradation. This takes place at a much lower rate, from which we can conclude that the primary target of free radicals is represented by lipids, proteins being the secondary one. Normally these signals should trigger endogenous antioxidant defence mechanisms, but this activation occurs at a much lower level than we expected. In accordance to literature data (23), we suppose that the malignant tissue is an oxidative stress inducer, but it also produces low-weight circulating proteins which contain sulphur and can mimic antioxidant activity, in order to prevent the activation of natural antioxidant systems. (24) Thus, we consider that when active oxygen metabolite production takes place in small concentrations, associated with hypoxia, the signals transmitted are towards modifying the phenotype under anaerobic conditions into one activating neovascularization, angiogenesis initiation, new cell growth and proliferation. The moment that this phase is overcome a new oxidative stress is installed, one potentially destructive for biomolecules essential to life, but also useful for further treatment, such as radiotherapy. (25)

Although hypoxia can physically lead directly to tumour cell resistance to radiotherapy (through decrease in free radical species, for example), a series of studies' results prove that hypoxia can have an impact on radioresistance by indirect paracrine mechanisms of stroma vascularization preservation. Lack of paracrine support signalling of tumour cells during irradiation (like in normoxic tumour regions) would leave endothelial cells more susceptible to dysfunction and death by irradiation. Thus, tumour regions in chronic hypoxia conditions, still viable, contain foci of resistant vessels which act as a target for post-irradiation paracrine signalling in the remaining tumour cells, which leads to new vascularization of the stroma. (26,27,28)

Regarding the relationship between the tumour tissue and oxygen metabolism we can state that it is a dual cause and effect one, but it is hard to determine whether the oxidative stress is a cause or an effect of the tumour presence.

Determining the oxidative stress parameters can offer data regarding the creation of an imbalance between oxygen reactive metabolite production and activation of natural intake systems, imbalance tilted towards the excessive production of active cytotoxic molecules (oxygen reactive species), future radiotherapy effectors, thus establishing a profile of patients who can develop resistance to postoperative radiation treatment.

Conclusions

In the present paper we have determined a series of oxidative stress parameters in a group of 30 patients with stage II cervical cancer, prior to surgical treatment, in order to establish their roles in the possible signalling mechanisms for shifting to an angiogenetic phenotype or in the oxidative destruction activity of tumour cell. All 30 pacients showed values more than 10 times higher than the normal ones, regarding the lipid degradation product. This level of lipid peroxidation is determined by an intense oxidative reaction, generated by the oxygen reactive species, which are produced by the oxygen metabolism, metabolism increased due to the tumour presence. Thus, the primary target of free oxygen radicals was represented by lipids, proteins being the secondary one. Normally these signals should trigger endogenous antioxidant defence mechanisms, but this activation occurs at a much lower level than we expected.

These tests can also be performed on tissue samples resulted during the surgical procedure, widening the clinical picture and thus helping avoid unnecessary radiation of patients who can develop resistance.

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