IMMUNOHISTOCHEMICAL ANALYSIS OF THE SKIN AFTER LOCAL ELECTRON IRRADIATION

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Abstract

Skin cancer is the most frequently diagnosed type of cancer among all malignant neoplasms. The decrease in mitotic activity and the death of intact keratinocytes arise due to the constantly renewing epithelium is highly sensitive to ionising radiation. Objective: immunohistochemical evaluation of the proliferative-apoptotic balance of keratinocytes, the fibrous component of the skin and the expression of pro-inflammatory and anti-inflammatory cytokines after single or fractional local electron irradiation.

Methods. Wistar rats (n=50) were taken and divided into groups: I – control (n=20), which were injected with saline; and experimental groups, local electron irradiation at doses: II – 8 Gy (n=10; single), III – 40 Gy (n=10, single), IV – summary dose 78 Gy (n=10; fractional; 13 Gy per day for 6 days). Results and Conclusions. 8 Gy and 40 Gy single local electron irradiation leads to a shift in the proliferative-apoptotic balance of keratinocytes towards their apoptosis, the activity of which is directly correlate with the dose of ionizing radiation, and 78 Gy summary dose in fractions leads to partial desquamation of the epithelium and inflammatory infiltration. In addition, a significant increase in the expression of type I and type III collagen fibers and the development of signs of radiation-induced skin fibrosis takes place against the background of 78 Gy fractional local electron irradiation. At the same time, after single 8 Gy and 40 Gy electron irradiation the described immunohistochemical changes were insignificant and directly correlated with the dose of ionizing radiation.

Introduction

Skin cancer is the most frequently diagnosed type of cancer among all malignant neoplasms [1]. Non-melanoma skin cancer is divided into basal cell carcinoma and squamous cell carcinoma through the development and spread of the oncological process, prognosis and morphological features [2].

Radiation therapy for non-melanoma skin cancer is used by oncodermatologists and radiobiologists in ineffective surgical treatment and as well as adjuvant or palliative therapy [3]. In this case, brachytherapy, hypofractionated and traditionally fractionated contact or surface irradiation with photons or X-rays are used, and the dose is selected individually, on average, for young people fractions in doses of 2–2.5 Gy, and for the elderly in doses of 3–5 Gy and more [4]. The damage to paratumoral tissues is often noted in the form of desquamation of the epithelium, necrosis of soft tissues, cartilage, and bone; pigmented changes, telangiectasias, fibrosis and skin atrophy, despite the relatively lite doses and fractionation modes [5, 6].

The fewest side effects are described in single works after fractional electron irradiation in total doses of 44 Gy and 54 Gy [7]. However, the mechanisms of post-radiation damage have not been fully elucidated, and studies of the cell cycle of keratinocytes are few and remain relevant.
The cell cycle of keratinocytes is regulated by proliferation proteins (Ki-67) and caspases, which responsible by apoptosis. The decrease in mitotic activity and the death of keratinocytes arise due to the constantly renewing epithelium is highly sensitive to ionising radiation.

The fibrosis of the skin structures is the most common complication of radiotherapy. An imbalance in the synthesis and metabolism of different types of collagen fibers plays an important role in the formation of fibrosis and is regulated by pro-inflammatory cytokines such as IL-1 and IL-6 and anti-inflammatory cytokines such as IL-4 and IL-10 [8, 9]. Type I collagen fibers give stability to the dermis, and type III collagen fibers provides elasticity and tensile strength. Degradation of type I and type III collagen fibers is initiated by matrix metalloproteinases-1 (MMP-1) and MMP-3, respectively [10, 11].

An increase in the synthesis of RNA of type I and III collagen fibers in the superficial layers of the dermis after high-dose irradiation for breast cancer was found using the in situ hybridization (ISH) [12]. An increase in the level of aminoterminal type I collagen propeptides-positive fibroblasts in irradiated skin are showed by immunohistochemical study. The authors have concluded that irradiation increases the expression of skin collagen genes, which are responsible for development fibrosis and thickening of the dermis [13]. On the contrary, the authors noted a change in the structure of collagen fibers to amorphous in recent studies using X-irradiation at a dose of 15 Gy. Temporary damage to the sebaceous glands and hair follicles without signs of inflammation, cell proliferation or fibrosis was found. It differs from previously described results in the skin from other parts of the body [14, 15]. So, the problem of radiation-induced fibrosis is still relevant.

Thus, the creation of experimental models for studying the proliferative-apoptotic balance of keratinocytes and the fibrous component of the skin will make it possible to assess the degree and depth of post-radiation damage to the skin after electron irradiation order to select optimal radiotherapy doses and improve methods for the prevention of radiation-induced fibrosis (RIF).

Objectives of the study: immunohistochemical evaluation of the proliferative-apoptotic balance of keratinocytes, the fibrous component of the skin and the expression of pro-inflammatory and anti-inflammatory cytokines after single or fractional local electron irradiation.

Methods

Experimental design. Wistar rats (n=50) were taken and divided into groups: I – control (n=20), which were injected with saline; and experimental groups, local electron irradiation at doses: II – 8 Gy (n=10; single), III – 40 Gy (n=10, single), IV – summary dose 78 Gy (n=10; fractional; 13 Gy per day for 6 days).

Ionizing radiation. Animals were exposed to local irradiation of the skin of the outer surface thigh (dose rate 1 Gy/min, energy 10 MeV and frequency 9 Hz, field size Ø 100 mm) using a linear accelerator (“NOVAC-11”, National Medical Research Radiological Centre, Obninsk, Russia).

All manipulations were carried in accordance with the International recommendations (EEC, Strasbourg, 1985), the European Convention for the Protection of Vertebrate Animals (EEC, Strasbourg, 1986), the Guidelines for Biomedical Research on the Care and Use of Laboratory Animals (ILAR, DELS), the Rules of laboratory practice and the Declaration of Helsinki (1964).

Histological examination. Skin fragments after fixation in a buffered formalin solution were prepared according to a standard protocol and stained with hematoxylin and eosin. The obtained histological micropreparations were analyzed in 10 fields of view of a light microscope.

Immunohistochemical study. Skin fragments after fixation for immunohistochemical analysis were prepared according to the standard protocol. As primary was used monoclonal antibodies to Ki-67 (ThermoFisher, Clone MM1, 1:100), p53 (ThermoFisher, Clone DO-7, 1:100), Caspase 3 (ThermoFisher, Clone 74T2, 1:500), Collagen-I (ThermoFisher, Clone COL-1, 1:200), Collagen-III (ThermoFisher, Clone FH-7A, 1:50), polyclonal antibodies to IL-1 beta (ThermoFisher, 1:100), IL-4 (ThermoFisher, 1:100), IL-6 (ThermoFisher, 1:100), IL-10 (ThermoFisher, 1:100), and secondary antibodies (HiDef Detection HRP Polymer system, Cell Marque, USA). Cell nuclei were counterstained with Mayer’s hematoxylin. The number of immunopositive cells was
counted in 10 randomly selected fields of view at ×400 magnification (in %). The amount of collagen fibers in the sections was measured using an image analysis computer program (BMI plus software, BumMi Universe Co.) and expressed as a percentage of the area occupied by collagen fibers in the upper dermis. Stained sections were scored using a modified numerical scale from 0 to 3 [16].

Microscopic analysis was performed using a video microscopy system (microscope Leica DM2000, Germany; camera Leica ICC50 HD).

Statistical analysis. The obtained data were processed using the computer program SPSS 12 for Windows statistical software package (IBM Analytics, USA). All data are expressed as mean ± standard deviation. A p-value < 0.05 was considered statistically significant.

Results

At microscopic examination the skin in the control group consisted of epidermis, papillary and reticular dermal layers and hypodermis, skin appendages and abundant hair follicles were clearly visualized (Figure 1A). Skin lesions of varying degrees were observed in the experimental groups (II – IV), which correlate with the radiation dose. A thickening of the epidermal basal layer in the hair follicles region with a slight delamination of the stratum corneum and intact appendages, the focal leukocyte infiltration, perivascular edema in the dermal papillary layer and dilated blood vessels with erythrocyte sludge was noted after 8 Gy single local electron irradiation (Figure 1B). Flattening and partial absence of the epidermal basal layer, smoothed dermal papillary layer, dilated blood vessels with erythrocyte sludge, absence of sebaceous glands, intact hair follicles and subcutaneous fat was noted in group III after 40 Gy single local electron irradiation. Microcavities of the epidermal-dermal junction, filled with desquamated epidermal cells and polymorphonuclear leukocytes were found (Figure 1C). The epithelium is partially absent, the dermal papillary layer is smoothed and intensely infiltrated with polymorphonuclear leukocytes, edema of the dermal reticular layer and hypodermis, collagen fibers are loosened, dilated blood vessels with endothelial detachment, aggregation and sludge of erythrocytes were noted after 78 Gy fractional local electron irradiation as well as the sebaceous glands and hair follicles are destructured (Figure 1D).

Immunohistochemical study. Nuclear staining with antibodies to Ki-67 in epidermal basal layer keratinocytes and interfollicular epithelial cells was noted. In contrast, melanocytes and Langerhans cells staining was not detected. A number of Ki-67-stained keratinocytes was decreased after 8 Gy (by 1.2 times) and 40 Gy (by 2.6 times) single local electron irradiation, as well as after 78 Gy fractional irradiation – by 5.9 times compared to the control was noted (Table 1, Figure 2).

A significant increase in caspase-3-stained keratinocytes was found after 8 Gy and 40 Gy single local electron irradiation by 3.4 and 6.6 times respectively, and after 78 Gy fractional irradiation by 11.6 times compared to the control (Table 1, Figure 2).

The number of p53-stained keratinocytes after 8 Gy and 40 Gy single local electron irradiation increased by 3.0 times and 5.3 times respectively and after 78 Gy fractional local electron irradiation by 7.3 times compared to the control group (Table 1, Figure 2).

Thus, there is a clear increase in the degree of keratinocyte apoptosis, which correlated with the dose of electron irradiation and the fractions was the most toxic to the healthy paratumoral tissues.

In the immunohistochemical study of skin samples the intensity of type I and type III collagen fibers staining were depending on the depth of post-radiation damage. The highest type I collagen fibers immunolabeling was observed after fractional irradiation compared to the control values (p<0.05). At the same time statistically significant decrease of type III collagen immunolabeling was found in the same group. In groups II (8 Gy) and III (40 Gy) the intensity of type I and type III collagens immunostaining changed slightly compared to the control (Table 2).

In addition, a slight type I collagen fibers average density decrease was revealed after 8 Gy single irradiation compared to the control group. And after 40 Gy single local electron irradiation its values are increase
A statistically significant increase after 78 Gy fractional local electron irradiation was observed compared to the control group.

A similar immunohistochemical pattern was observed when staining skin fragments with antibodies to type III collagen with a significant increase in their average density in group IV compared with the control (Figure 3c, 3d). An increase in the number of IHC-positive for collagens I and III types of fibroblasts was noted, most pronounced in the group of fractional irradiation in SOD 78 Gy was noted in the skin samples of the experimental groups after irradiation.

A slight immunolabeling of keratinocytes of the basal layer of the epidermis, as well as the dermis showed the immunohistochemical study of skin fragments of the control group with antibodies to pro-inflammatory (IL-1β, IL-6) and anti-inflammatory cytokines (IL-4, IL-10). An increase in the degree of immunostaining of both pro- and anti-inflammatory cytokines was observed after local electron irradiation in single doses of 8 Gy and 40 Gy with increasing dose the intensity of labeling increased compared to the control. An imbalance of the studied markers of the inflammatory response was found: high levels of immunolabeling with antibodies to pro-inflammatory cytokines (IL-1β, IL-6) and a sharp decrease in the intensity of staining with antibodies to anti-inflammatory cytokines (IL-4, IL-10) compared with the values of the control group after fractional electron irradiation at a total dose of 78 Gy (Figure 4).

Discussion

This study is devoted to the immunohistochemical evaluation of impaired keratinocyte proliferation, skin collagen synthesis, the degree of radiation-induced fibrosis and the expression of inflammatory cytokines after 8 Gy and 40 Gy single local electron irradiation and 78 Gy fractional local electron irradiation. Radiation therapy is based on the concept that actively proliferating abnormal cells are more sensitive to ionizing radiation and cannot regenerate as efficiently as healthy ones. During a few hours there are formation of DNA double-strand breaks (direct effect), reactive oxygen species (ROS) and other radicals that damage DNA and other cellular components (for example, cell membranes, proteins and lipids; indirect effect) [17-19]. Cell death occurs by apoptosis or necrosis with the release of damage-associated molecular patterns (DAMPs): heat shock proteins, HMGB1, hyaluronan fragments, etc. [20, 21].

Some authors noted the death of keratinocytes in the epidermal basal layer after 3-5 Gy single γ-irradiation [22, 23]. However, in our study an 8 Gy single local electron irradiation leads to weak epidermal pathomorphological changes. A slight decrease in the density of collagen fibers found after 8 Gy single local electron irradiation is also explained by the relatively low damaging ability of this dose [24], at which microscopic analysis of the skin showed only a partial thickening of the basal layer and delamination of the stratum corneum of the epidermis. At the same time, the dermis remained intact, which explains the absence of differences in collagen metabolism in this group compared to the control, since most of the collagen fibers and fibroblasts that produce them are located mainly in the papillary dermis [25].

A decrease in the density of microvessels in the skin, leading to tissue ischemia and, thereby, significantly reducing the rate of wound healing after 25 Gy X-irradiation was found in another study. In addition, the authors noted an increase in the expression of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6), the release of cell adhesion molecules, which accelerated the migration, adhesion and exudation of leukocytes and induced the migration of macrophages, neutrophils and monocytes, which leads to a local inflammatory response to radiation injury [26]. The polymorphonuclear infiltration of the skin found at various doses of single local electron irradiation can be explained by a similar mechanism the development of inflammation, the key role in which is played by the imbalance of pro-inflammatory and anti-inflammatory cytokines. These changes in the inflammatory response are most pronounced after fractional local electron irradiation. Thus, immunohistochemical analysis showed an increase in both pro-inflammatory (IL-1, IL-6) and anti-inflammatory (IL-4, IL-10) cytokines after 8 Gy single local electron irradiation. This was also more pronounced after 40 Gy single local electron irradiation, however, the balance of cytokines was preserved. Thus, probably that damage after 8 Gy and 40 Gy is compensated and can be repaired, and after 78 Gy irradiation decompensation occurs. Pro-inflammatory cytokines predominate over anti-inflammatory
cytokines. Disruption of compensatory defense systems also occurs and all these changes lead to development of unrepairable acute (radiation-induced dermatitis, RID) and late (radiation-induced skin fibrosis, RIF) post-radiation complications.

Some authors have noted necrosis of keratinocytes, which pathogenetically associated with ischemia due to destruction of the blood vessels, as well as damage to the sebaceous and sweat glands and hair follicles after 45 Gy photons irradiation [27, 28]. On the contrary, in our study hair follicles were preserved at all levels of the skin and the sebaceous glands were absent, probably due to their greater radiosensitivity after 40 Gy single local electron irradiation. This fact indirectly confirms the concept of a “light” effect of electron irradiation on the skin appendages.

In the study, the most pronounced skin damage was observed after electron fractions with minor damage to the vascular wall, while other types of irradiation (for example, photons, X- and γ-rays) at similar fractionation doses led to its complete destruction [6].

The molecular mechanisms of radiation-induced cell death are still poorly understood. The revealed imbalance between proliferation and apoptosis of keratinocytes, including those caused by irradiation, leads to Bcl-2 deactivation and p53 induction. This is accompanied by a decrease in the cell pool due to the modulation of GSK3-, ERK- and Ras/Raf/MEK-1 signaling pathways [29, 30].

A decrease in the number of Ki-67-positive keratinocytes, which is associated with the direct toxic effect of ionizing radiation on actively proliferating cells, in which the destruction of cell membranes and the structure of macromolecules (DNA, RNA, proteins, lipids, etc.), as well as modulation of MAPK, PI3K, and NFκB signaling pathways, proteins of the ErbB family, and disruption of cellular respiration at the level of the electron transport chain in mitochondria was observed after 8 Gy and 40 Gy. All these processes leads to an imbalance of the antioxidant and prooxidant systems [31]. In addition, the observed inhibition of keratinocyte differentiation is associated with damage to such signaling pathways as: PI3-kinases/Akt and Ras/Raf/MEK-1, as well as glycogen synthase kinase-3 (GSK-3) and extracellular signal-regulated kinase (ERK) [29].

Modulation of the activity of the above cascades leads to chromatin condensation and DNA fragmentation, i.e. apoptosis, confirmed by an increase in the number of caspase-3- and p53-positive cells. Apoptosis is activated via extrinsic and intrinsic pathways and caspase-3 is responsible for the terminal phase. The cell cycle regulator is the proapoptotic protein p53, which also was increased [32].

It should be noted a decrease in the number of mitotically dividing generations of epidermal basal layer keratinocytes was observed after single electron irradiation [33, 34]. Thus, the results of the IHC study showed a shift in the proliferative-apoptotic balance towards keratinocyte apoptosis, which is most pronounced in the fractional group compared to single electron irradiation [31]. So, unlike other types of ionizing radiation, electrons show the smallest depth of skin damage.

Many studies have reported skin fibrosis after inflammation in response to injury, thermal and chemical burns and radiation. In turn, inflammation (RID) is described as a consequence of ionizing radiation, which contributes to the destruction of macromolecules and cell membranes with the formation of reactive oxygen species (ROS) and oxidative stress develops. This leads to inflammatory infiltration and disruption of the connective tissue structure (i.e., a decrease in the density of collagen fibers) [35, 36]. For example, ROS destroy the endothelium of small-caliber blood vessels, triggering a cellular inflammation [19]. This leads to the activation of pro-inflammatory cytokines, an increase in the expression of which was found in all irradiated groups. However, in response to the ROS release an increase in antioxidant system enzymes has been reported, which aim to capture and destroy ROS, thereby reducing skin damage. The key role belongs to metalloproteinase-9, which is responsible for the remodeling of extracellular matrix components and is regulated by endogenous tissue inhibitors of metalloproteinases and TGF-β1, the relationship of which in the skin after irradiation has not been studied enough [14, 37].

After the initial reaction to radiation damage the cells of the extracellular matrix begin to regenerate the
damaged structures. The production of collagen fibers, i.e. fibrosis is the most common outcome of this process. Moreover, in some cases, this process can probably proceed uncontrollably as a result of disruption of regulatory mechanisms after irradiation at high doses with total damage to the deep layers of the skin, which was confirmed by the results of our study after 78 Gy fractional electron irradiation (group IV) and partially after 40 Gy single electron irradiation (III group). This is consistent with the opinion of other authors [13].

According to the results of an immunohistochemical study, an increase in the dose and frequency of exposure led to hyperactivation of collagen synthesis due to the intensification of signaling pathways, probably responsible for fibrosis [15, 35]. In our study an increase in immunolabeling for type I and type III collagen fibers in skin fragments after high doses of single local electron irradiation was revealed, and 78 Gy fractional irradiation led to the most active production of collagen fibers, the density of which in this group was significantly higher compared to the control. This result of electron irradiation is called radiation-induced fibrosis [15].

After 8 Gy single local electron irradiation the risk of developing radiation-induced fibrosis is minimal, since the deep layers of the skin, in which fibroblasts are located and collagen fibers are synthesized, remain intact with a high degree of probability. However, according to the results of histological and immunohistochemical studies an intensification of inflammation and collagen formation and signs of skin radiation-induced fibrosis as an outcome of radiation-induced dermatitis with an increase in the radiation dose were observed. They were most pronounced in the fractional electron irradiation group and an important role was played by the destruction of the skin deep layers with a violation of the regulation of collagenogenesis. Probably, it was also related with the duration of impact of the damaging factor.

Conclusions

8 Gy and 40 Gy single local electron irradiation leads to a shift in the proliferative-apoptotic balance of keratinocytes towards their apoptosis, the activity of which is directly correlate with the dose of ionizing radiation, and 78 Gy summary dose in fractions leads to partial desquamation of the epithelium and inflammatory infiltration. In addition, a significant increase in the expression of type I and type III collagen fibers and the development of signs of radiation-induced skin fibrosis takes place against the background of 78 Gy fractional local electron irradiation. At the same time, after single 8 Gy and 40 Gy electron irradiation the described immunohistochemical changes were insignificant and directly correlated with the dose of ionizing radiation.

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References


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