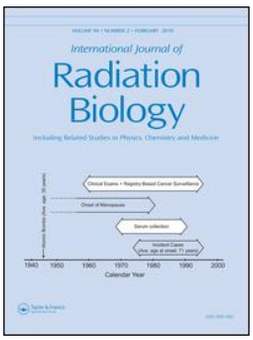

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ORIGINAL ARTICLE

Fluorescence of radiation-induced tissue damage

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ABSTRACT

Purpose: The aim of this study was evaluating changes in the photosensitizer fluorescence in vivo in the radiation-induced damage area in comparison of intact areas with a simultaneous assessment of changes both in blood parameters and in histological data.

Materials and methods: The study was conducted in white outbred SHK mice ($n = 21$). Their right hindlimbs were irradiated with a dose of 25 Gy after the intraperitoneal injections of photosensitizer 'Photosens'. Fluorescence intensity was traced in vivo by a laser diagnostic system for seven weeks. Simultaneously, histological examination of the damaged areas and blood tests were performed.

Results: An increased intensity of the laser-induced fluorescence of the photosensitizer 'Photosens' in the damaged areas, compared to the intact symmetrical ones was observed. Laboratory blood tests and histological examination showed changes that may indicate the occurrence of inflammation.

Conclusion: Enhanced intensity of the exogenous fluorescence of the photosensitizer in the radiation-induced inflammation of noncancerous tissues was observed. The obtained results may potentially affect an interpretation of the results of intraoperative tumors navigation that have been previously irradiated and can be used for selection of an individualized dose fractionation algorithm in radiology.

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Laser fluorescence spectroscopy; inflammation; radiation damage; photosensitizer accumulation; in vivo

Introduction

Laser fluorescence spectroscopy (LFS) in vivo with photosensitizers is currently used in photodynamic therapy and in surgical oncology for an intraoperative assessment of tumor boundaries and in a number of other sections of medicine (Rogatkin et al. 2012; Toh et al. 2015; Koch and Ntziachristos 2016; Zhang et al. 2017). The LFS technique of intraoperative navigation is based on the assumption that cancerous tissues do cumulate the photosensitizers more effectively than healthy tissues. Some studies have shown that the decrease in extracellular pH promotes the enhanced accumulation of the photosensitizers (Friberg et al. 2003; Čunderlíková et al. 2005; Mojzisova et al. 2007). The vast majority of malignancies are characterized by increased glucose uptake and glycolysis. This metabolic shift results in enhanced production of lactic acid which leads to decreased pH (Longo et al. 2016) and, perhaps, exactly it may consequently lead to higher tumors fluorescence in comparison to normal tissues.

It is also well known that inflammation is associated with a pH shift to the acidity (Yen et al. 2009). According to some published data, this phenomenon is related to elevated tissue temperature and to increased metabolic activity (Willerson 2004). Therefore, we assumed that local inflammation could lead to the accumulation of photosensitizer. Our hypothesis has been confirmed in previous studies demonstrating an increased laser-induced fluorescence of the photosensitizer 'Photosens' in tissues with thermally induced and mechanically induced inflammation (Petritskaya et al. 2015; Guseva, Rogatkin, et al. 2016).

Sometimes surgical treatment of malignancies is preceded by radiation therapy (Wzietek et al. 2013; Swallow et al. 2015; Erlandsson et al. 2017). There are some published data that LFS-based intraoperative navigation after preliminary irradiation of a glioma is quite difficult due to the nonspecific accumulation of a photosensitizer acid in the area of radiation-induced necrosis (Goriainov et al. 2014). Perhaps, this nonspecific photosensitizer accumulation was a consequence of the radiation-induced inflammation arouse in tissues that follows from the data on magnification of a photosensitizer accumulation in the inflamed area (Petritskaya et al. 2015; Guseva, Rogatkin, et al. 2016).

However, articles we have found on this topic are sporadic. Therefore, the investigation of the accumulation of different types of photosensitizers in different types of irradiated tissues is relevant. This information can be extremely useful at surgical fluorescent navigation. As the first step, in the framework of this study, we explored an

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accumulation of the photosensitizer 'Photosens' in healthy irradiated tissues.

Also, the radiation damage degree assessment by LFS can become potentially useful for individualized fractioning of the radiation dose in oncology in the future, in terms of prevention of local advanced inflammation response. Radiation therapy is one of the most commonly used methods of malignant tumors treatment due to its efficiency (Joiner and Van der Kogel 2009). The need for a dose fractionation to improve the radiation therapy effectiveness, as well as to minimize the side effects, was described as far back as 1934 (Coutard 1934). Up to date, the development of new dose-fractionation schemes to enhance the therapeutic effect remains topical (Haviland et al. 2013; Perkó et al. 2017). Local inflammatory reactions are common complications of the radiation therapy. They are associated with severe pain that may result in interruption of a radiation treatment cycle (Gruber and Dörr 2016; Mallick et al. 2016). Despite the fact that a pause in the radiation therapy course reduces its effectiveness due to the tumor cells repopulation between irradiations (Kim and Tannock 2005), however in a number of cases doctors have to break the course due to the development of acute local reactions (Tchernyi et al. 2005). A noninvasive technique that could quantify local tissue changes in the early post-radiation period would be helpful for a radiologist to predict potential complications and to individually select the radiation dose.

The impact of single large radiation doses is widely used in animal studies and allows better understanding of the irradiation effects than the use of fractional irradiation schemes (Kozin et al. 2012). These studies are especially relevant, since single dose therapy or several large radiation fractions are being tested in hospitals more often (e.g. stereotactic therapy/ablative radiotherapy or radiosurgery) (Lo et al. 2010; Tipton et al. 2011; Kozin et al. 2012). In our previous pilot experiment some increased accumulation of the photosensitizer in the irradiated at a dose of 15 Gy hind of laboratory mice compared to the intact hind have been shown (Guseva, Kulikova, et al. 2016). However, inflammation that was registered in the pilot experiment was not fairly expressed for its reliable identification by the LFS method. It was suggested that increased accumulation of the photosensitizer is associated with inflammation induced by exposure to ionizing radiation. To confirm or disprove this hypothesis, we decided to increase the dose of ionizing radiation up to 25 Gy.

These data may potentially allow a reconsideration of principles of intraoperative navigation in tumorous areas that have been previously exposed to radiation therapy, as well as for modification of current approaches to the assessment of their boundaries with the purpose to increase navigation accuracy. Also, it can be used for selection of an individualized dose fractioning algorithm in radiology.

Problems of a correct data interpretation in the fluorescence diagnostics of tumors as well as the developed techniques application are rather comprehensive and need to be resolved on different levels, including cellular. This study is dedicated to the examination of a single irradiation influence on the ability of irradiated normal tissues to accumulate the photosensitizer 'Photosens'. Tumor tissues were not examined at this stage.

Materials and methods

The study was performed in SHK mice ($n = 21$) (purchased from 'Pitomnik Stolbovaya', RF). Their bodyweight at a study entry was 26–33 g. To exclude any gender-associated differences, only female animals were included into the study.

All animal experiments complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines; they were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU (European Union) Directive 2010/63/EU for animal experiments and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). An independent ethics committee № 5 approved the trial on 12 May 2016.

Right hindlimbs of the experimental animals were irradiated at the dose of 25 Gy with a short-focused X-ray generator Wolf T-160 (WOmed GmbH, St Gangloff, Germany). The radiation source in this system is an X-ray tube with a voltage of 80 kV, a current amperage of 15mA, a half-value layer – 1 mm of aluminum, and a field diameter of 50 mm (Figure 1). At the given parameters of irradiation, the maximum in the dose distribution falls on the external surface of the foot. The contralateral limbs were left intact.

Often when studying local inflammatory reactions of laboratory animals, the hindlimb is the one that is being examined. The choice was due to the pairing of the organ, which makes it possible to use an identical contralateral region as an intact region. The relative anatomical 'detachment' of the limb makes it possible to clearly determine the localization of irradiation without the need to place 'marks' on the body, which allows for a higher accuracy of the study. Also, choice of this area for investigation allows to achieve less variability of the volume of the affected tissue in different animals. In addition, the bones rich in red bone marrow, sensitive to irradiation, are located in the mice'

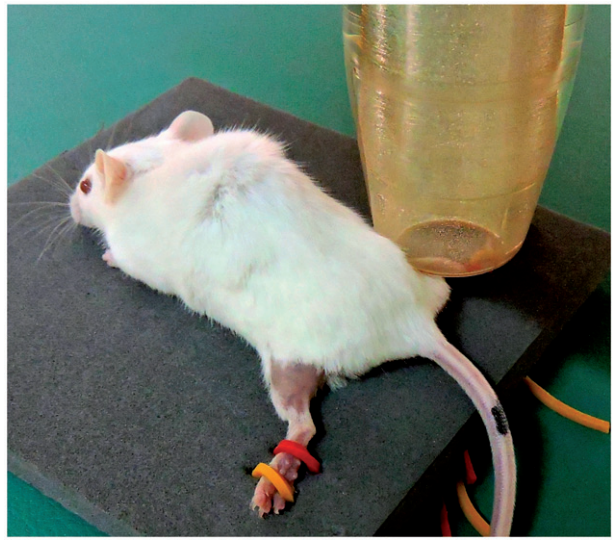

Figure 1. Position of the irradiated region.

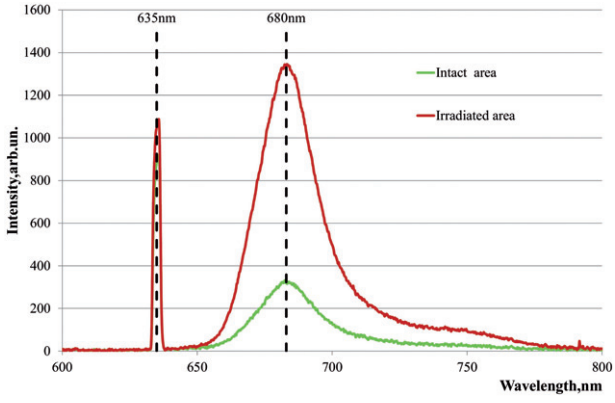

Figure 2. Examples of secondary radiation spectra of irradiated and contralateral regions (16th day after irradiation).

hindlimbs. Therefore, it is possible to assess not only local radiation damage, but also changes in hemopoiesis after the irradiation.

To immobilize the area of irradiation, all mice were narcotized and fixed on a scaffold (Figure 1). Aluminum hydroxide trisulfophthalocyanine photosensitizer 'Photosens' (Niopik, RF) in the dosage of 2 mg/kg that is used in photodynamic therapy (Kholodtsova et al. 2014) was injected intraperitoneally to all animals right before irradiation.

The maximum 'Photosens' fluorescence is observed at the wavelength $\lambda_f = 680$ nm. Among the endogenous fluorophores in the wavelength range 650–700 nm, only porphyrins fluoresce, and the main contribution is made by protoporphyrin IX, whose fluorescence wavelength maxima are $\lambda_{f1} = 630$ nm and $\lambda_{f2} = 690$ nm (Croce and Bottiroli 2014). Nevertheless, the fluorescence of endogenous substances is many times lower than the fluorescence of the 'Photosens', therefore, in spectra obtained in the measurement, the second maximum is not observed (Figure 2), thus, we disregard the influence of endogenous fluorophores in this work.

The laser-induced fluorescence intensity was measured in vivo by LFS in hindlimbs. Measurements were done on the skin surface directly above the damaged area and above the symmetrical area on the contralateral intact limbs.

All measurements of fluorescence intensities were repeated for seven weeks. The value before the irradiation was taken as the baseline. All measurements were performed with the use of the multifunctional laser diagnostic system 'LAKK-M' (Lazma, RF) in the 'Fluorescence' operation regime. Excitation of tissue fluorescence was made in the continuous wave mode at the wavelength 635 nm (a semi-conductor laser). Power of the laser radiation on a distal end of the optical fiber probe (on a surface of tissues) was around 5 mW. Laser radiation was delivered to the damaged area surfaces by the multimode optical fiber. Probing and receiving fibers are silicon ones with a diameter of 100 μm. A distance between them is 1mm. Registration of the fluorescence flux was carried out in the waveband 650–680 nm by the built-in fiber optic spectrometer with the CCD detector, which is included in the system design. Fluorescence intensity was measured at 680 nm – in a maximum of the fluorescent spectrum of the used photosensitizer 'Photosens'. Subsequently, the intensity at this wavelength will be called 'fluorescence intensity'.

In order to exclude the individual characteristics of a photosensitizer accumulation in the animal's tissues and to evaluate only changes in the dynamics of fluorescence caused by damage, we used the relative index of inflammation intensity $\mu(\lambda_f)$, which was calculated as follows (Rogatkin et al. 2012; Guseva, Rogatkin, et al. 2016):

$$\mu(\lambda_f) = I_f(\lambda_f)/I_{f0}(\lambda_f), \quad (1)$$

where I_f is the fluorescence intensity from the inflamed area, I_{f0} is the fluorescence intensity from the contralateral area, and λ_f is the fluorescence wavelength (for Photosens, $\lambda_f = 680$ nm).

Statistical analysis was done with Statistica 13.2 (Dell Inc., Tulsa, OK, USA) software. The required sample size was calculated for one-sample Student's t test. The null-hypothesis (H_0) was that the value of $\mu(\lambda_f)$ does not differ from 1. For statistical power calculation, the standard deviation for $\mu(\lambda_f)$ of 0,7 and arithmetic mean of 1,5 were chosen. It was calculated that at least 18 animals were required for 80% power of the study. Quantitative data were analyzed with calculation of arithmetic means and 95% confidence intervals of means Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA). The null-hypothesis (H_0) '$\mu(\lambda_f)$ values does not differ from 1' was tested with the one-sample Student's t test. Normality of the distribution was tested with Shapiro-Wilk W test.

Animals were withdrawn from the experiment for histological examination of the damaged limb at Days 0, 3, 8, 13, 20, 36, 45, and 49 (two mice at each time point). For this purpose we took one animal with a high fluorescence level and the other with a low one as a way not to obtain animals only with a low inflammation intensity or a high one at the end of the experiment.

Histological examination was performed according to the standard protocol; 4 μm paraffinized sections were stained with hematoxylin eosin. The limbs were examined in full; sections were done at various levels perpendicular to the femoral bone axis. Hematology tests were done in all experimental animals at Days 0, 3, 8, 13, 20, 36, 45, and 49 to assess systemic response to radiation injury.

Results

Figure 2 shows examples of spectra detected from the surface of the intact and irradiated legs. The obtained spectra are characterized by the presence of two maxima corresponding to the backscattering peak (635 nm) and to the fluorescence of the 'Photosens' (680 nm).

An increase in the mean relative index of the fluorescence intensity after local irradiation indicating an active accumulation of the photosensitizer in the irradiated area was found. The group mean value of $\mu(\lambda_f)$ is shown in Figure 3. As can be seen from the figure, it has a number of maxima (at Days 16, 22, and 30) and minima (at Days 20, 28, and 45); 95% confidence intervals are also given on the diagram. The difference between $\mu(\lambda_f)$ and 1 was significant from Day 3 up to Day 41 at $p < .05$ (one sample t test). The number of animals

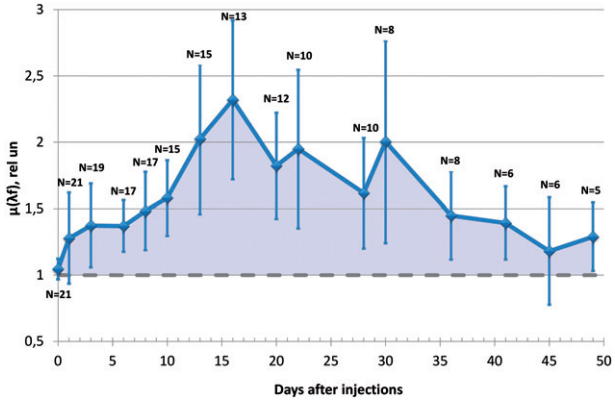

Figure 3. Changes of the mean relative index of the fluorescence intensity $\mu(\lambda_f)$ during the study. Vertical bars represent 95% confidence intervals. The null-hypothesis (H_0) '$\mu(\lambda_f)$ values does not differ from 1' was tested with the one-sample Student's *t* test.

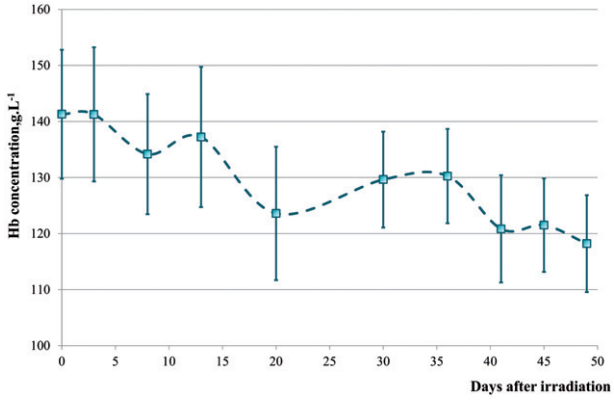

Figure 4. Changes in the average value of Hb concentration at a dose of 25 Gy. Vertical bars represent standard deviations.

decreased eventually due to their gradual elicit from the experiment for histological examination. Beyond that, four of them died for other reasons, such as anesthetic mortality, etc.

A systemic response to the radiation injury was confirmed by hematology tests in the experimental animals. A gradual, step-like decrease in hemoglobin level after radiation injury was also demonstrated throughout the observation period (Figure 4).

After irradiation, an increase in white blood cell counts with a local maximum at the Day 8 was observed. After that, the absolute number of white blood cells decreased, the minimum value was recorded on Day 30 followed by a rise on the 45th day. It was accompanied by 'mirror' changes in the level of band neutrophils – a decrease in the 7th day, a maximum value on the 30th day, a minimum value on the 45th day (Figure 5).

The results of the histological examination showed that in some animals, there were reliable histological signs of radiation dermatitis. It was observed that animals had mild inflammatory changes at the Day 1. At Day 3, there was a dramatic nonspecific inflammatory response, while at Days 8 and 13, specific abnormalities were seen typical for radiation dermatitis (as lichenoid dermatitis). At the end of the study (Day 49), the predominant morphological signs

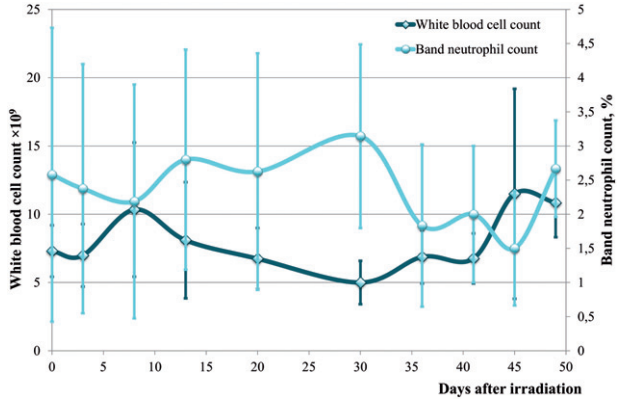

Figure 5. Changes in mean absolute white blood cell counts (left axis) and in band neutrophil counts (right axis) after local radiation at a dose of 25 Gy. Vertical bars represent standard deviations.

were nonspecific chronic dermatitis with reactive hyperplasia of epidermis, dermal fibrosis and regenerative changes (Figure 6).

Discussion

Thus, our data indicate that irradiated areas do actively accumulate the photosensitizer 'Photosens'. Taking into account previous results of our pilot experiments (Guseva, Kulikova, et al. 2016), we can conclude now that the relative index of the fluorescence intensity (Equation (1)) goes up with higher irradiation doses. After irradiation at the dose of 15 Gy, the maximum mean relative index of the fluorescence intensity was 1.3 relative units (Guseva, Kulikova, et al. 2016), whereas after the dose of 25 Gy, it reached 2.3 relative units. In addition, the fluorescence intensity index has a multi-peak curve, which may reflect the dynamics of local radiation damage.

When analyzing histological data, it was shown that in all animals studied, the histological pattern revealed a mild inflammatory reaction in the first day after the radiation exposure, then in some animals nonspecific inflammatory changes were detected on day 3 and the development of specific anomalies characteristic of radiation damage at 8th and 13th days, that is, in the period of the intensive 'growth' of $\mu(\lambda_f)$. At the end of the study, on the 49th day, when there was a decrease in $\mu(\lambda_f)$, a morphological picture of nonspecific chronic dermatitis with reactive hyperplasia of the epidermis and fibrosis in the dermis prevailed. As mentioned above, in previous studies we have shown that an increase in photosensitizer's accumulation is detected in the region of local thermally and mechanically induced inflammation, possibly increased fluorescence in the area of radiation exposure is also due to inflammatory changes.

Regarding the changes in the clinical analysis of blood, the gradual decrease in the level of hemoglobin attracts attention. It is known that hematopoietic cells are sensitive to radiation damage, and the hindlimb of mice is rich in red bone marrow. Thus, it can be explained by the injury of the bone marrow, the erythrocyte progenitor cells and by the suppression of hematopoiesis with the subsequent gradual occurrence of anemia. Such a decrease in the hemoglobin

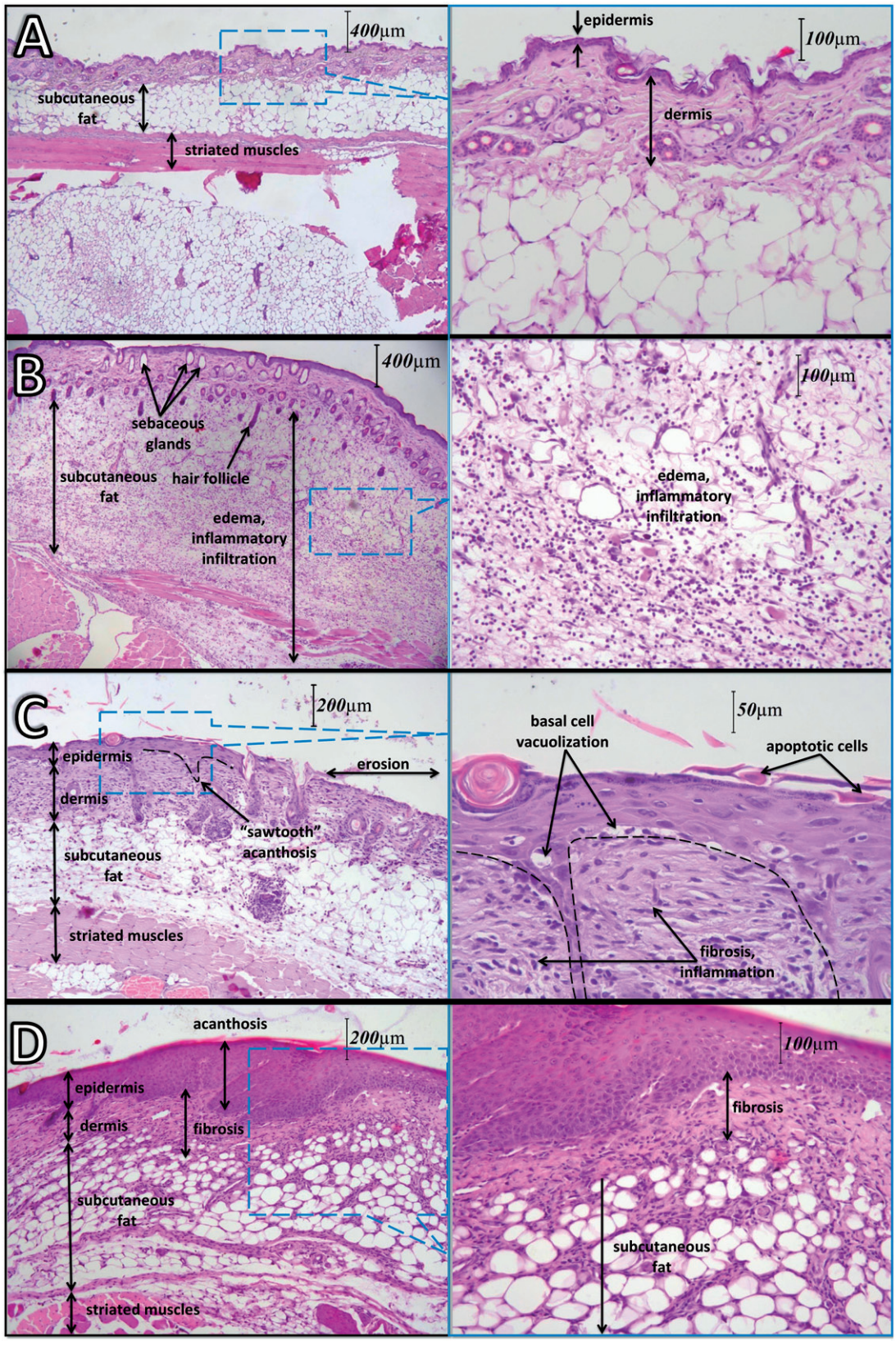

Figure 6. (A) Contralateral unirradiated limb. Normal tissue. (B) On the Day 3 after irradiation. Acute injury with the prevalence of a nonspecific inflammatory response, edema, advanced inflammatory infiltration with neutrophils, lymphocytes, histiocytes. (C) On the Day 8 after irradiation. Lichenoid dermatitis with a destroyed basal layer of the epidermal cells, keratinocyte apoptosis, basal cell vacuolization, atrophy and formation of erosions, sawtooth acanthosis. Dermal fibrosis and inflammatory infiltration with lymphoplasmatic cells. (D) On the Day 49 after irradiation. Regenerative signs: reactive epidermal hyperplasia, acantosis, advanced fibrosis. The images also show normal anatomical structures of the skin: sebaceous glands, hair follicles, pilosebaceous units. Staining with hematoxylin-eosin.

level can illustrate the significant effect of the applied dose of local irradiation on hemopoiesis.

After irradiation, an increase in white blood cell counts with a local maximum at the Day 8. It could be interpreted as inflammation-induced leukocytosis (this coincides with the histological signs of advanced inflammation at the Day 8). Further decline in white blood cell counts can be explained by their response to ionizing irradiation. In this situation, two alternative processes may take place in the experimental animals: one is the decrease in white blood cell counts related to a radiation injury of the bone marrow and the other is their increase due to the inflammatory response. In addition, a decrease in the level of white blood cells is accompanied by a 'mirror' increase in band neutrophils. This can be explained by a compensatory increment in a relative number of immature forms of white blood cells against the background of the decrease in the level of white blood cells induced by the radiation damage. White blood cell counts are restored by the Day 45 with an over-regeneration reaction. The analysis of band neutrophils, that is immature leukocytes, is of special interest. The increase in this parameter represents a shift of the differential counts to the left that is typical for inflammatory response. The release of immature cells into the blood stream indicates enhanced neutrophilic regeneration that can be explained by both as the classical response to inflammation and, in this case, as a compensatory response to radiation injury of the white cell hemopoietic lineage. As can be seen from Figure 3, the maximum band neutrophil count is registered at Days 13 and 30 corresponding to the high value of the fluorescence coefficients.

As mentioned above, the evaluation of the ability of irradiated nontumorous tissues to accumulate photosensitizers may be important for a competent interpretation of the results of fluorescent intraoperative tumors navigation subjected to radiotherapy. The data obtained indicate a possible increase in the accumulation of a photosensitizer in nontumorous tissues after irradiation, these data, in our opinion, can contribute to a deeper understanding and new interpretation of the results of fluorescent diagnostics of tumors subjected to preliminary irradiation.

There is a clear need to continue research in this direction; more accurately and in detail to study the dynamics of accumulation of a photosensitizer in nontumorous radiation-affected tissues and compare it with that of a tumor. Further studies may contribute to increasing the specificity of fluorescence diagnostics of irradiated tumors.

In this paper, we have shown that a noninvasive method such as LFS can diagnose radiation-induced changes in normal tissues, which in turn can become the basis for the development of new techniques that allow to correct irradiation schemes depending on individual tissue reactions to treatment. In the long term, such techniques may allow minimizing severe long-term complications of radiotherapy.

In addition, it is known (Coussens and Werb 2002) that inflammation is a critical component of a tumor progression. We would also like to note that there is still no unified point of view explaining the mechanism of the selective photosensitizer accumulation in tumor tissues (Mycek and Pogue 2003). As we have been shown in our previous studies (Guseva, Rogatkin, et al. 2016) that the photosensitizer accumulation in the inflammation region (radiation-induced, as well as mechanically or thermally induced) may hypothetically be one of the reasons of the photosensitizer accumulation.

Conclusion

This study was intended to evaluate reactions induced by irradiation of healthy tissues of hindlimbs of laboratory mice and accumulation of the photosensitizer in the injured areas. We found the increased fluorescence of the photosensitizer in the areas of radiation-induced damage more than 2 times at Days 13, 16, and 30. In previous studies with a smaller dose of local irradiation, an increase in the fluorescence intensity index reached only 1.3 times. We also obtained results of histological examination of the injured areas and of hematology tests in the experimental animals that reflected the intensity and the dynamics of the inflammation. Maximal values of the mean band neutrophil count reflected the run of inflammatory processes also were observed on Days 13 and 30. It was shown that the results of LFS in vivo go in parallel with the results of both hematology tests and histological examinations and prospectively may reflect the dynamics of the local damage.

Our study was aimed at evaluation of the in vivo laser-induced fluorescence of the irradiated tissues with photosensitizers. In our opinion, its data could be prospectively useful for interpretation of the results of intraoperative navigation in the presurgically irradiated areas. Also, the results obtained open new perspectives to develop quantitative criteria measuring the degree of radiation response. In future, this may help to individualize algorithms of radiation doze fractioning for tumor radiation therapy.

Note also that this investigation can contribute to an understanding of the causes of the photosensitizer redistribution between tumorous and nontumorous tissues exposed to irradiation. Perhaps in the future, researchers will focus on seeking ways to differentiate tumors from inflamed tissues by optical methods that can be combined technically with the LFS. We hope, the data obtained will provide a background for further studies in this area and will focus attention on the raised problem.

Disclosure statement

The authors report no conflicts of interest.

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