Hypoxia

Local hypoxia in oral mucosa (mouse) during daily fractionated irradiation – Effect of pentoxifylline

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Purpose: A significant reduction of radiation-induced oral mucositis by systemic application of pentoxifylline has been demonstrated in a mouse tongue model. However, the underlying mechanisms remain unclear. The present study focuses on the development of local hypoxia in mouse tongue during daily fractionated irradiation and a potential modulation by pentoxifylline.

Materials and methods: Daily fractionated irradiation with 5 × 3 Gy/week (days 0–4, 7–11) was given to the snouts of mice. Groups of 3 animals per day were sacrificed between day 0 and 14. Pentoxifylline (15 mg/kg, s.c.) was administered daily from day -5 to the day before the mice were sacrificed. The expression of intrinsic hypoxia markers HIF-1α and GLUT1 in the epithelium of the lower tongue surface was analysed by immunohistochemistry in 3 animals per day; the percentage of positive epithelial cells and the staining intensity were analysed as endpoints.

Results: Compared to untreated control tissue, fractionated irradiation resulted in a progressive increase in the expression of both hypoxia markers. This effect was significantly reduced by pentoxifylline.

Conclusion: An early onset of local hypoxia occurs during fractionated irradiation in mouse tongue epithelium. The effect is markedly reduced by the mucoprotective agent pentoxifylline, suggesting a mucositis-promoting role of hypoxia; this, however, deserves further investigation.

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Abstract

Oral mucositis is a severe and often dose-limiting early side effect of radio(chemo)therapy of advanced head-and-neck malignancies [1–4]. So far, no biology-based mucoprotective approach has been introduced into clinical practice; current supportive care strategies are mainly symptomatic [5,6]. Radiation-induced oral mucositis is based on the impairment of proliferation in the germinal epithelial compartment, which results in epithelial hypoplasia and consequently in epithelial denudation [7,8]. However, these processes are preceded and accompanied by various inflammation-associated changes, which are likely to interact with the epithelial radiation response proper [9,10]. Also, these alterations may result in the manifestation of local hypoxia [11]. Pentoxifylline (PTX) is an unspecific phosphodiesterase inhibitor applied in clinical practice to treat chronic vascular insufficiencies [12]. PTX exhibits a variety of biological effects, including increased red blood cell pliability, thereby promoting perfusion [13]. PTX significantly reduced radiation-induced oral mucositis in the mouse tongue model [14]. Also, systemic administration of PTX was suggested to prevent oral mucositis in patients receiving bone marrow transplantation [15].

The present study was therefore initiated to (1) characterize changes in the expression of intrinsic hypoxia-markers (HIF-1α, GLUT1) during fractionated irradiation and (2) assess the effect of PTX on the expression of these markers.

Material and methods

Animals and housing

In the present experiments, mice of the inbred C3H/Neu strain from the breeding facility of Medical Faculty Carl Gustav Carus, Dresden, Germany were housed under specified pathogen-free conditions with controlled temperature (21–24 °C) and humidity (30–50%). An automated light programme provided a 12/12-h light/dark rhythm, with lights on from 06:00 am to 06:00 pm.
Maximum ten animals were kept in size 3 Macrolon® cages on saw dust bedding (Sniff 3/6, Altrogge, Lage, Germany) with free access to standard mouse diet (Altromin 1326, Altrogge, Lage, Germany) and filtered city tap water from standard perspex drinking bottles.

Irradiation technique

The technique for irradiation of oral mucosa has been described in detail elsewhere [16,17]. In brief, percutaneous irradiation of the entire snout was performed with an YXLON MG325 device (Yxlon International X-ray GmbH, Hamburg, Germany), operated at 200 kV with a tube current of 20 mA. The animals were guided into plastic tubes (inner diameter 28 mm). A conical hole in a perspex block at the front end of the tubes served for standardized positioning of the snouts. The back ends of the tubes were closed to prevent withdrawal of the animals. Eight animals were irradiated simultaneously. The bodies of the mice were shielded with 6 mm of lead equivalent MCP-96 (HEK Medizintechnik, Lübeck, Germany). The treatment field encompassed the snouts including the entire tongue. The dose homogeneity between the individual snout irradiation fields was ±3%.

Experimental design

Daily fractionated irradiation with 5 × 3 Gy/week was applied over 2 weeks (days 0–4, 7–11). The study comprised two experimental arms: irradiation alone and in combination with PTX. PTX was administered at a dose of 15 mg/kg subcutaneously from day 5 until the day before the mice were sacrificed; on irradiation days, the drug was given one hour before irradiation. In both arms, groups of 3 animals were sacrificed, prior to irradiation (controls) and at intervals of 2–14 days after the start of daily fractionation. Their tongues were excised at the base for further investigations. Three untreated and unirradiated mice served as control group.

Histological preparation and analysis

The tongues were fixed in 4% paraformaldehyde for 24–48 h, cut along the median line, and subjected to routine paraffin embedding. Sections of 3 μm were mounted on Superfrost® plus charged glass slides (Gerhard Menzel GmbH, Braunschweig, Germany) and dried at 37 °C overnight. Subsequently, the sections were dewaxed in xylene and rehydrated through a graded alcohol series. After heat-mediated antigen retrieval (20 min, citrate buffer, pH 6.0), endogenous peroxidase activity was blocked (10 min, 3% hydrogen peroxide). The sections were then incubated with normal goat serum (1:200) using a Vectastain® ABC Kit (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature, followed by overnight incubation at 4 °C with the primary antibodies against HIF-1α (Abcam, Cambridge, MA, USA; Cat No. 2185; rabbit polyclonal) at a concentration of 1:400 or GLUT1 (Abcam, Cambridge, MA, USA; Cat No. 652; rabbit polyclonal) at a concentration of 1:600. A second section on the same slides was incubated with the same concentration of rabbit IgG (Dianova GmbH, Hamburg, Germany; Cat No. 011-000-003) and served as a control. The secondary antibody was added for 1 h at room temperature. Afterwards, sections were incubated with avidin–biotin complex solution for 1 h at room temperature. The enzyme reaction was visualized by 3,3-diaminobenzidine (DAB) substrate (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). Nuclear counterstaining was performed with haematoxylin (5 min). Then, slides were dehydrated in a graded alcohol series, cleared in xylene and coverslipped.

Analysis was performed with an Olympus light microscope, using a 400× magnification. HIF-1α and GLUT1 expression was analysed separately in the germinal and functional layer of the epithelium of the lower tongue surface. The fraction of cells with expression of HIF-1α (nuclear) or GLUT1 (membrane, as well as the relative staining intensity of both proteins were evaluated. The staining signal intensity was scored semi-quantitatively with an arbitrary score from 0 (no signal), 1 (weak), 2 (intermediate) to a maximum of 3 (strong). Visual evaluation of the two endpoints, i.e. 1) number of HIF-1α and GLUT1 positively stained epithelial cells and 2) the respective staining intensity, was performed in at least 5 microscopic fields. From the staining intensity values, a mean value was calculated for each animal. This procedure was applied despite the fact that a non-parametric score was applied.

Statistical analysis

For statistical analysis, the SPSS statistical software (SPSS Inc., Chicago, IL, USA) was used. Mean values calculated for each animal, which then served to calculate the mean and standard error for each experimental group. The analysis of variance (one-way ANOVA) was used to test for the significance of a difference between the mean values. A p-value of <0.05 was regarded statistically significant.

Results

Representative immunohistochemical expression of HIF-1α and GLUT1 in unirradiated and untreated control specimen and on day 6 and 12, respectively, is presented in Fig. 1. The coloured version of Fig. 1 can be found in the supplementary.

HIF-1α

In control tongues, nuclear HIF-1α was found in 42.7% of germinal epithelial cells and 70.1% of functional epithelial cells. During irradiation, a progressive increase of the HIF-1α expressing cells was found in the germinal layer of the epithelium, with a peak of 85.8% on day 12 (Fig. 2A). In the functional layer, the fraction of HIF-1α expressing cells increased to 88.6% within the first 4 days and subsequently remained at elevated levels (Fig. 2B).

The HIF-1α staining intensity in the germinal layer increased from an average value of 1.1 arbitrary units (a.u.) in control epithelium to a maximum average value of 2.5 (a.u.) on day 10 (Fig. 2C). In functional cells, the HIF-1α staining intensity increased from an initial average value of 1.7 a.u. in control specimen to 2.6 a.u. on day 8 and 10, followed by a subsequent decline (Fig. 2D).

With additional PTX treatment, the fraction of HIF-1α expressing germinal cells was lower compared to irradiation alone at all time points (Fig. 2A). A substantial effect was observed on the HIF-1α staining intensity, which remained within the range of unirradiated control epithelium, except on days 6 (1.6 a.u.) and 12 (1.4 a.u.), as shown in Fig. 2C. In functional epithelial cells, PTX treatment decreased the fraction of HIF-1α positive cells significantly to 48.7% on day 2 and 69.1% on day 4 compared to 84.7% and 88.6%, respectively. Afterwards, no significant difference to irradiated epithelium was found (Fig. 2B).

GLUT1

In control epithelium, 0.6% and 0% of germinal and functional epithelial cells, respectively, showed membrane-associated expression of GLUT1. During irradiation a progressive increase in the number of GLUT1-positive germinal cells to 70.3% on day 14 was observed (Fig. 2E). No expression was found in the functional compartment. The staining intensity of GLUT1 in the germinal
layer increased progressively from 0.3 a.u. in controls to a maximum of 2.6 a.u. on day 12 (Fig. 2F).

PTX reduced the fraction of GLUT1 expressing cells significantly on all time points investigated, except on days 10 and 14. (Fig. 2E). Also, the staining intensity remained lower than in only irradiated animals (Fig. 2F).

**Discussion**

During radio(chemo)therapy of head-and-neck tumours, virtually all patients experience some degree of oral mucositis, the majority develops a severe, confluent reaction [18,19]. Oral mucositis significantly impacts on the patient's quality of life, is a major socioeconomic factor [2,4,20] and sometimes necessitates unplanned treatment breaks, which reduce the tumour control probability [21,22]. So far, no biology-based prophylactic or treatment strategy was introduced into clinical practice. PTX was suggested as a mucositis ameliorating agent [5,15] and also showed a significant mucoprotective potential in preclinical studies [14]. PTX is an unspecific phosphodiesterase inhibitor with various effects, including modification of rheological properties [12]. Pathological and functional consequences of fractionated irradiation with and without additional PTX treatment on mouse oral epithelium will be characterized in a separate manuscript (Gruber et al., in preparation). The present study was initiated to investigate changes in the expression of the intrinsic hypoxia markers HIF-1α and GLUT1 in mouse oral epithelium during daily fractionated irradiation alone and in combination with administration of PTX. Under normoxic conditions, HIF-1α is rapidly degraded. During hypoxia, HIF-1α is stabilized and translocates into the nucleus, where it binds to hypoxia response elements in the promoter region of its target genes and induces target gene transcription, among them the gene for glucose transporter 1 (GLUT1) gene [23]. The latter is integrated into the cell membrane, where it facilitates cellular glucose uptake [24,25].

**Effect of fractionated irradiation alone**

**HIF-1α**

A marked fraction of HIF-1α positive cells was found in both epithelial layers in control samples. Irradiation induced a progressive increase in the fraction of nuclear HIF-1α expressing germinal cells and their respective HIF-1α staining intensity in both epithelial compartments. GLUT1 expression was limited to the germinal epithelial layer and only induced upon irradiation. PTX treatment diminished GLUT1 membrane-associated expression at all time points. Scale bar: 50 μm.

**GLUT1**

GLUT1 was only found in the germinal epithelial compartment. Fractionated irradiation also increased the fraction of GLUT1 positive cells, most pronounced in the second week. The present results exclusively refer to membrane-associated immunohistochemical staining of GLUT1. Interestingly, also an atypical, nuclear signal was observed (data not shown) during fractionated irradiation. These observations are currently subject to further investigations.

Fractionated irradiation induced a marked increase in the expression of both hypoxia markers studied. This indicates the induction of local hypoxic conditions in the epithelium. However, increased perfusion as measured by computed tomography has been demonstrated in head-and-neck cancer patients with clinically manifest oral mucositis [26]. Moreover, early radiation-induced vasodilatation is frequently found in the skin and mucosae of radiotherapy patients [27] and was also described in the present animal model [9]. It may be assumed that vasodilation and increased perfusion are associated with increased oxygenation of the tissue. Yet, an increased vascular diameter, abnormal vessel
permeability and vascular leakage in combination with inflammatory changes in the vascular environment may result in a decrease in the oxygen supply to the epithelium, particularly the germinal layer, as observed in the present study. The biological relevance of this assumed local hypoxia, e.g., its likely interaction with other local processes, however, currently remains unclear and deserves further investigation.

**Effect of PTX**

PTX at least partly inhibited the radiation-induced changes in both hypoxia marker proteins. Hypothetically, this potentially contributes to the mucoprotective capacity of the drug; e.g., through increased erythrocyte pliability and/or a reduction of inflammatory processes. Hypoxia induces inflammatory conditions, and vice versa inflammation is frequently associated with hypoxia [11]. These aspects, however, clearly require further studies.

**Conflict of interest statement**

S. Gruber, D. Hamedinger, E. Bozsaky, M. Schmidt, K. Wolfram, J. Haagen, B. Habelt, M. Puttrich and W. Dörr state that there are no conflicts of interest.

**Compliance with ethical guidelines**

All institutional and national guidelines for the care and use of laboratory animals were followed. All experiments were performed with approval by the respective authorities (Landesdirektion Sachsen, file No. 24D-9168.11-1/2006–14).
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2015.03.024.

References


