

• BRIEF REPORTS •

Enhancement of leukocyte adhesion after percutaneous irradiation in rats with hepatocellular carcinoma

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Abstract

AIM: To evaluate the effects of percutaneous radiation on leukocyte-endothelium interaction (LEI) in experimental hepatocellular carcinoma (HCC).

METHODS: Twelve ACI rats underwent HCC-inoculation, six of which on day 12 received low-dose external radiation and six did not. After 12 h intravital microscopy was performed.

RESULTS: LEI was significantly reduced in tumor tissue. However, irradiation of liver sinusoids and tumor tissue with 6 Gy led to a significant activation of leukocyte adhesion in the tumor with a marked increase of the proinflammatory cytokine TNF- α .

CONCLUSION: The findings indicate that the immunological tumor-endothelial barrier can be overcome by external irradiation.

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Key words: Radiation; Hepatocellular carcinoma; Immune response; Animal model

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INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) is increasing worldwide, and represents the third most common cause of cancer-related death^[1]. Although complete surgical resection is the primary goal, long-term survival is often limited by local recurrence or distant metastases of the tumor.

Because of this challenge, there is a need to develop and evaluate new treatment options in HCC. At present, most patients are diagnosed when palliation with controversial survival benefits is the sole option^[2]. Innovative therapeutic strategies are currently tested in HCC, including new biological target-based drugs, cyclooxygenase inhibitors, and gene therapy are currently tested in HCC^[3].

Physiological and pathological vascularization events are not equivalent among organ systems, and the unique behavior of each system needs to be taken into account when evaluating angiogenic therapies^[4]. Leukocyte-endothelium interaction (LEI) and migration could represent a worthwhile target for novel immunological treatment strategies as it represents the first and fundamental step in the immune cascade leading to tumor cell recognition and possible rejection. Without leukocyte adhesion to malignant tumor endothelium, there will be no migration into the tumor. That malignant tumors protect themselves by inhibiting leukocyte adhesion to their endothelial surface is a common phenomenon in pancreatic and other cancers^[5-8].

LEI consists of the rolling of leukocytes along the vascular wall, and firm adherence of leukocytes to endothelial cells under certain conditions. This phenomenon is processed by cell adhesion molecules. Although significant progress has been made in understanding the underlying mechanisms of LEI, a therapeutic approach by enhancing the development of effective leukocyte infiltration in tumors is not established.

Irradiation of healthy tissues is known to upregulate endothelial adhesion molecules^[9-11]. We hypothesized that a percutaneous single dose radiation of 6 Gy could enhance LEI within the tumor and induce intratumoral migration of leukocytes.

MATERIALS AND METHODS

All experiments were performed with permission of the government authorities and in accordance with the German legislation on laboratory animal experiments (Regierungspräsidium Karlsruhe, Germany). Male ACI rats weighing 230.0 ± 39.2 g (VAF-Plus, Harlan-Sprague Dawley, Indianapolis, USA) were used in all experiments. Animals were anesthetized with ketamine/pentobarbital (Ketanest S, Parke-Davis, Berlin, Germany and Narcoren, Merial, Hallbergmoos, Germany). The left carotid artery and jugular vein were cannulated for blood pressure measurement and application of FITC-labeled albumin and rhodamine 6G.

Experimental design

On day zero, intrahepatic tumor implantation of Morris

hepatoma A3294 was performed in 12 ACI rats^[12]. The recipient rats were anesthetized and the left liver lobe via a small midline incision was prepared for tumor implantation. Five million Morris hepatoma tumor cells (5 μ L) were injected in subcapsular position. On d 12, six animals underwent a percutaneous single dose radiation with 6 Gy under general anesthesia. After 12 h the animals underwent laparotomy and intravital fluorescence microscopy was performed to determine tumor vessel diameter, red blood cell velocity (RBV) and leukocyte adherence^[13]. Values were compared to control animals without irradiation.

All animals were killed at the end of videomicroscopy and the whole liver was harvested for histopathological investigations. Serum specimens were taken at the end of experiments for enzyme analysis and TNF- α values.

Videomicroscopy

Intravital videomicroscopy was used according to the epiillumination technique reported by Menger *et al*^[14]. In general anesthesia the left liver lobe was exteriorized after relaparotomy. A Leitz fluorescence microscope (Leitz GmbH, Wetzlar, Germany) was used. In the presence of different excitation filters (wavelength 450-490 and 530-560 nm) visualization of FITC-labeled erythrocytes (Fluorescein isothiocyanate Isomer 1, Sigma, St. Louis, USA) and leukocytes with rhodamine 6G (0.02 mg/kg body-weight, Sigma) was possible. For contrast enhancement of plasma, FITC-labeled albumin was administered intravenously during experiments (50 mg/kg body-weight, Sigma). The microscopy was videotaped and off-line analysis was performed using a computer-assisted processing system^[15].

The following parameters were assessed in ten randomly selected tumor areas and fields of healthy liver tissue. RBV was measured using the frame to frame method offline. Volumetric blood flow (V_b) was visualized after intravenous injection of FITC-labeled erythrocytes and analyzed offline. Determinants were erythrocyte velocity and vessel diameter (D) using the following equation: $V_b = 15 \times V_c \times \pi \times D^2$ ^[16]. LEI described the flow behavior of white blood cells and differentiated between low-affinity leukocytes (roller) moving with less than 66% of RBV or adhering for less than 30 s to the endothelium and high-affinity leukocytes (sticker) adhering for more than 30 s to the endothelium surface.

Hemodynamics

Blood gas analysis and monitoring of heart rate and mean arterial blood pressure were performed via the cannulated left carotid artery at the beginning of experiments, 30 min after the onset of videomicroscopy and 2 h after microscopy (ABL 5, Radiometer GmbH, Willich, Germany).

Cytokine measurement (TNF- α)

On d 13, blood samples of radiated animals and controls were taken after videomicroscopy for TNF- α measurement using a standardized ELISA kit (Pharmingen, USA).

Histology

One part of the harvested liver was fixed in buffered formalin and prepared for staining with hematoxylin and

eosin to confirm tumor presence.

Statistical analysis

The data were expressed as mean \pm SD and compared between groups by Wilcoxon-Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

There were no significant differences between the study groups in mean arterial blood pressure and blood gas analysis during intravital microscopy.

Control hemodynamics and blood gases were maintained at physiological levels throughout the experiments.

Vessel diameter and basal RBV were comparable in hepatic tumor tissue and healthy liver tissue (Tables 1 and 2). There was a homogenous but not significant increase in volumetric blood flow in both groups (Table 3).

The number of high-affinity leukocytes was comparable in tumor tissue and healthy liver tissue ($P > 0.05$) (Figure 1). After percutaneous low-dose irradiation (6 Gy), high-affinity LEI was significantly enhanced in tumor tissue and sinusoids ($P < 0.05$) (Figure 1).

TNF- α levels were significantly elevated after radiation ($P < 0.05$) (Figure 2).

DISCUSSION

The results of the current study indicate that LEI decreases significantly in tumor tissue under basal conditions, but this can be overcome by low-dose external radiation. We have previously shown that tumor-associated endothelial cells have a suppressed expression of ICAM-1 compared to endothelial cells from healthy liver^[13]. Thus, only the basal expression of endothelial adhesion molecules is decreased in the tumor vasculature, but the possibility of inflammation-mediated upregulation is not hampered. The homogeneous LEI in

Table 1 Red blood cell velocity (RBV) (mm/s, mean \pm SD)

| RBV | Healthy liver | Liver cancer |
|-----------|----------------|-----------------|
| Controls | 1.49 \pm 0.3 | 1.85 \pm 0.12 |
| Radiation | 1.88 \pm 0.1 | 1.93 \pm 0.14 |

Table 2 Vessel diameter in liver tumor tissue and healthy hepatic parenchyma (μ m, mean \pm SD)

| Vessel diameter | Healthy liver | Liver cancer |
|-----------------|-----------------|-----------------|
| Controls | 34.5 \pm 3.8 | 36.0 \pm 3.71 |
| Radiation | 35.6 \pm 2.82 | 36.5 \pm 0.92 |

Table 3 Volumetric blood flow pattern in liver tumor tissue and healthy liver parenchyma (nL/s, mean \pm SD)

| Blood flow | Healthy liver | Liver cancer |
|------------|-----------------|-----------------|
| Controls | 0.25 \pm 0.07 | 0.29 \pm 0.02 |
| Radiation | 0.31 \pm 0.03 | 0.35 \pm 0.05 |

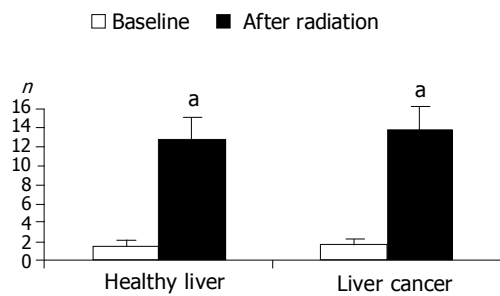


Figure 1 High-affinity LEI with a significant increase after radiation in both study groups (mean \pm SD, ^a $P<0.05$).

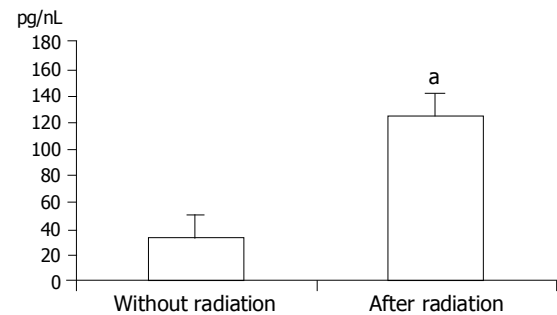


Figure 2 Significant elevations of TNF- α values (pg/mL) after percutaneous irradiation (^a $P<0.05$).

liver sinusoids and tumor vessels after radiation indicates that endothelial cells can be activated probably by unspecific radiation-induced inflammation with a marked increase of proinflammatory cytokine TNF- α .

Radiotherapy with or without transarterial embolization and/or percutaneous ethanol injection appears effective in controlling HCC and can prolong survival^[17] although this is still controversial. External beam radiation is rarely used as a single modality therapy as it has been shown that more than 50 Gy would be required to kill HCC cells. However, this radiation dose is commonly associated with radiation-induced hepatitis and liver failure when the whole liver is treated^[18,19]. In contrast, high-dose conformal radiotherapy, including proton irradiation, has shown significant responses and acceptable toxicities by excluding the non-tumorous volume of the liver from the target volume. Local radiotherapy combined with transarterial catheter embolization (TACE) has also been investigated as a means of enhancing tumor control, because TACE has a limited effect on portal vein tumor thrombus and pericapsular invasion of the tumor. This approach may provide response rates of 50%^[20,21] and overall survival benefit^[22].

If tumor tissues are associated with tumor infiltrating lymphocytes at a high density or with sinus histiocytosis in its regional lymph nodes, good postoperative survival rates for cancer have been reported^[23,24]. Involvement of an anti-tumor effect via cellular immunity, humoral immunity or via cytokines produced by the cancer cells has been discussed. TNF- α plays a critical role in the immune defense against tumor growth. By a regional infusion of the cytokine TNF- α and interferon- γ a significant reduction in tumor growth can be described in an animal model^[25].

We focused on microcirculatory parameters and the course of TNF- α after radiation. Quantification of the LEI and determination of the proinflammatory cytokine TNF- α showed a significant increase after external single-dose radiation. These findings are in line with those of earlier studies, indicating that inflammation-mediated upregulation of adhesion molecules in tumor endothelium is possible^[6,26].

Angiogenetic factors are capable of inducing a state of endothelial cell anergy. After activation by inflammatory cytokines there is a suppressed response of tumor endothelial cells compared to endothelial cells from normal tissue in human umbilical vein and human renal cell carcinoma^[27]. This state is induced at the protein level (expression) and at the functional level (adhesion) and may serve as a tumor-

protecting mechanism by impairing the development of efficient leukocyte infiltration into tumors^[28,29].

In conclusion, percutaneous irradiation of the liver has an impact on the LEI in endothelium of HCC and healthy liver. This observation is important because it may allow to overcome an important immune escape mechanism of malignant endothelium, which is downregulated by adhesion molecules. These findings have to be further evaluated using additional immunologic effector cell stimulation in immunotherapeutic studies in HCC.

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