Diminished Leukocyte-Endothelium Interaction in Tumor Microvessels

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Abstract

Leukocyte–endothelium interaction in vivo consists of the rolling of leukocytes along the vascular wall and, under certain conditions, their adherence to endothelial cells. In a rat tumor microcirculation model (mammary adenocarcinoma implanted in rat skinfold window chamber), we demonstrated that this interaction, measured as flux of rolling leukocytes and density of adhering leukocytes, was significantly reduced in tumor microvessels compared to normal microvessels, both under control conditions and during inflammation induced by N-formylmethionylleucylphenylalanine (1 μM), bacterial lipopolysaccharide (1 μg/ml), or tumor necrosis factor α (500 units/ml). We also measured the blood flow shear rate in the tumor and normal microvessels and found that the difference in shear rate between the two types of microvessels could not account for the differences in leukocyte–endothelium interaction. The diminished leukocyte–endothelium interaction in tumors under various stimulated conditions suggests that a number of adhesion molecules may not be expressed properly on tumor endothelial cells.

Introduction

Leukocytes interact with venular endothelial cells in vivo by rolling along the venular wall and, under inflammatory conditions, adhering to the vessel wall. This interaction affects the status of blood flow in the tissue, since adhering leukocytes can greatly increase the vascular resistance (1). Rolling and adhesion of leukocytes precede their transendothelial migration from the vascular space into the interstitial space, where they fulfill their immunological functions. These aspects of L/E interaction have direct relevance to tumor therapy: (a) blood flow regulation in tumor tissues plays a significant role in different modalities of cancer therapy (i.e., radiotherapy, chemotherapy, and hyperthermia) (2); (b) methods of cancer immunotherapy using specific classes of leukocytes would depend on the ability of such cells to transverse the vessel wall and enter tumor parenchyma (3).

In a study for separate purposes (4), we observed that leukocyte rolling and adherence appeared to be reduced in tumor microvessels compared to those in normal microvessels of rat dorsal skinfold chamber preparations. The purpose of the current study was to verify the initial observation by testing the hypothesis that the interaction between leukocytes and endothelial cells is significantly less in tumor than in normal microvessels. Since L/E interaction is known to be mediated by various adhesion molecules expressed on leukocytes and endothelial cells (5, 6), we investigated L/E interaction during inflammation mediated by mediators known to stimulate different types of adhesion molecules. Specifically, we used fMLP to stimulate the adhesion molecules on leukocytes and LPS or TNF-α to stimulate those on endothelial cells.

Materials and Methods

Animal Model. We used the dorsal skinfold window chamber implanted in Fischer rats (7). Tumor microcirculatory preparations were made by placing 0.1 mm² of rat mammary adenocarcinoma (R3230Ac) in the skinfold chamber. Tumor bearing rats were used 7 to 10 days after surgery, when the tumors grew to 3 to 4 mm in diameter and were well vascularized.

Chemicals. Acridine orange (Sigma Chemical Co., St. Louis, MO) was dissolved in saline at 0.5%. fMLP (1 μM; Sigma), LPS (1 μg/ml; Sigma), and murine TNF-α (500 units/ml; Genzyme, Cambridge, MA) were dissolved in PSS before each experiment.

Leukocyte Visualization. The window chamber was placed underneath a microscope (Zeiss photomicroscope III) equipped with epifluorescence capability. Leukocytes were stained with i.v. injected acridine orange, which was intermittently delivered via an infusion pump (Model 341B; Sage Instruments, Boston, MA). Leukocytes were best visualized by injecting a small bolus of acridine orange at 0.3 ml/min for 5 s immediately before each observation. The preparation was observed with a ×40 objective. The behavior of leukocytes in individual microvessels was recorded by a silicon intensified tube camera (Model CZ400; Hamamatsu Photonicns, Hamamatsu City, Japan) on videotape, which was analyzed after experiments.

Experimental Procedure. In each experiment, a rat bearing a skinfold window chamber was anesthetized with sodium pentobarbital (40 mg/kg i.p.). The femoral artery and vein were cannulated for blood pressure measurement and i.v. injection of acridine orange, respectively. The rat was then placed on a temperature controlled microscopic stage. One coverglass of the skinfold window chamber preparation was removed to expose the tissue to superfusion products for various experimental conditions. The rest of the experiment was conducted according to one of the following two procedures.

In the first procedure, the exposed tissue in the window chamber was initially superfused with PSS (pH 7.4, 32°C) as the control condition, during which approximately 5 randomly selected vessels were recorded. Each recording lasted for 1 min. Then fMLP (pH 7.4, 32°C) was continuously applied to the preparation. Ten min after the onset of fMLP superfusion, another 5 to 10 vessels were recorded.

In the second procedure, after the coverglass of one side of the window chamber was removed, LPS or TNF-α was applied topically to the preparation. The treatment solution filled only the bottom half of the window chamber (approximately 1 mm deep); the top 1 mm was filled with mineral oil to prevent water evaporation and heat loss. Finally, a thin glass coverslip was placed on top of the window chamber to limit air diffusion into the preparation. After 4 h of treatment, leukocyte behavior in individual microvessels of tumor and normal tissues was recorded, as described above. A set of control experiments was also performed in which the treatment solution was only PSS.

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3The abbreviations used are: L/E, leukocyte-endothelium; fMLP, N-formylmethionylleucylphenylalanine; LPS, bacterial lipopolysaccharide; TNF-α, tumor necrosis factor α; PSS, physiological salt solution; vWF, von Willebrand Factor; ICAM, intercellular adhesion molecule.
Quantitation of L/E Interaction. The recorded vessels were from both tumors and normal tissues surrounding tumor regions. Only postcapillary venules were selected. For each vessel, the numbers of rolling leukocytes \(N_{\text{rolling}}\) passing a certain point in the vessel and of adhering leukocytes \(N_{\text{adhering}}\) within a vessel segment of known length \(L\) were counted for a 30-s observation period. Vessel diameter \(D\) and the velocity of up to 10 free-flowing leukocytes were also measured. A rolling leukocyte was defined as one that migrates along the vessel wall and is clearly dissociated from the bulk blood flow. An adhering leukocyte was defined as one which stays stationary during 30 s of observation. Two quantities were calculated to characterize the extents of leukocyte rolling and adherence, respectively. They were (a) the average flux of rolling leukocytes \(F_{\text{rolling}}\):

\[
F_{\text{rolling}} = \frac{N_{\text{rolling}}}{30 \text{ s}}
\]

and (b) the average density of adhering leukocytes \(D_{\text{adhering}}\):

\[
D_{\text{adhering}} = \frac{N_{\text{adhering}}}{\pi \cdot D \cdot L}
\]

A pseudo shear rate \(\gamma_s\) was also calculated for each vessel as

\[
\gamma_s = \frac{8V_L}{D}
\]

where \(V_L\) is the mean velocity of free-flowing leukocytes.

Statistical Analysis. Data of same vessel type were pooled for each experimental condition. Differences in \(F_{\text{rolling}}\) and \(D_{\text{adhering}}\) between normal and tumor tissue vessels were assessed using the general linear model. Due to the nonnormality of \(F_{\text{rolling}}\) and \(D_{\text{adhering}}\) data, the Poisson error term model was fit to the data. In the analysis, adjustment was made for variations in vessel shear rate \(\gamma_s\) and rat differences. The purpose of adjusting for \(\gamma_s\) is to account for the possible effect of blood flow shear force on L/E interaction. The significance of differences in \(F_{\text{rolling}}\) and \(D_{\text{adhering}}\) between the two vessel types was ascertained by the likelihood ratio test. Estimates for differences between normal and tumor vessels (on the log scale) of both mean \(F_{\text{rolling}}\) and \(D_{\text{adhering}}\) were obtained. These estimates were exponentiated to give the ratio of the mean for normal tissue to that for tumor tissue. This analysis was carried out with the GLIM statistical package (8).

Results

Fig. 1 shows normal and tumor microvessels, respectively, under fMLP stimulated conditions. While the normal vessels are heavily decorated with adhering leukocytes, the tumor vessels are almost devoid of them.

In Fig. 2, \(F_{\text{rolling}}\) and \(D_{\text{adhering}}\) are plotted for both normal and tumor tissues, under control and fMLP-stimulated conditions. Twelve rats were used for this set of experiments. Under control conditions, mean \(F_{\text{rolling}}\) was higher in the normal tissue than in the tumor, although the difference between the two tissues was not statistically significant. \(D_{\text{adhering}}\) was, however, significantly higher in the normal tissue than in the tumor tissue. Stimulating the preparation with fMLP increased the flux of rolling leukocytes in the normal tissues but not in the tumor, thus \(F_{\text{rolling}}\) became significantly higher in the normal tissue. fMLP increased \(D_{\text{adhering}}\) in both the normal and the tumor tissues, but the magnitude of this increase was much greater in the former. Thus the difference in \(D_{\text{adhering}}\) between the two tissue types was further enlarged by fMLP stimulation. These results suggest that, under both control and fMLP stimulated conditions, leukocyte-endothelium interaction was significantly less in tumor tissues in comparison to normal tissues.

L/E interaction was also examined in preparations treated with LPS or TNF-\(\alpha\). The results of these experiments are presented in Fig. 3. Under control conditions (3 rats), both \(F_{\text{rolling}}\) and \(D_{\text{adhering}}\) were significantly higher in normal than in tumor tissues. Treating the preparation with LPS (5 rats) or TNF-\(\alpha\) (4 rats) did not change \(F_{\text{rolling}}\) in the normal tissues but increased it in tumors and increased \(D_{\text{adhering}}\) in both normal and tumor tissues. However, the significant differences remained in \(F_{\text{rolling}}\) and in \(D_{\text{adhering}}\) between normal and tumor tissues. Thus, under LPS or TNF-\(\alpha\) stimulated conditions, L/E interaction was also significantly reduced in tumor tissues in comparison to normal tissues.

The leukocyte-endothelium interaction is known to be affected by hemodynamics within the blood vessels; higher shear stress on the endothelial surface would tend to reduce leukocyte rolling and remove adhering leukocytes (1, 9). Therefore we tested whether the detected difference in leukocyte-endothelium interaction between the two tissues could be accounted for by a difference in hemodynamics. For each microvessel, a pseudo shear rate \(\gamma_s\) was used as an approximate indication of
LEUKOCYTE-ENDOTHELIUM INTERACTION IN TUMOR

Control fMLP
Treatment

Fig. 2. Comparison of leukocyte rolling and adherence frequency between normal and tumor tissues under control and fMLP stimulated conditions. A. average flux of rolling leukocytes (Frolling): B, average density of adhering leukocytes (Dadhering). Numbers in parentheses, number of vessels examined. *, statistically significant difference, after each parameter was adjusted for 75, between tumor and normal tissues under the same experimental condition (/> 0.05). Bars, SEM.

shear stress.5 The average γ₅ values for normal and tumor tissues are listed in Table 1 under each experimental condition. It can be seen that while there was no difference in γ₅ between normal and tumor tissues under fMLP and its control conditions, γ₅ was significantly higher in tumor than in normal tissues under LPS, TNF-α, and their control conditions. Thus the lower L/E adherence in tumor microvessels under these latter conditions could be due to the higher shear stress existing in these vessels. To assess the effect of shear stress on leukocyte rolling and adherence, the differences in Frolling and Dadhering between normal and tumor tissues were statistically analyzed with a model which allows adjustments of Frolling and Dadhering for γ₅ and compares each parameter between the two tissues for comparable γ₅. This analysis indicated that, even after adjustments for the pseudo shear rate γ₅, there were still significant differences in both parameters under most of the experimental conditions as shown in Figs. 2 and 3. Therefore, the detected difference in the L/E interaction between normal and tumor tissues cannot be accounted for merely by differences in hemodynamics.

Discussion

In this study, we compared the extent of leukocyte-endothelium interaction between normal and tumor tissues in the rat skinfold window chamber microcirculation preparation. This interaction was characterized by leukocyte rolling and adherence on the wall of postcapillary venules. We found that, under both control and inflammatory conditions, the level of L/E

Table 1 Pseudo shear rates (mean ± SE) and ratios of the means under different experimental conditions

| Experimental condition | Pseudo shear rate (s⁻¹) | Ratio of means⁵
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Normal vessels</td>
<td>Tumor vessels</td>
</tr>
<tr>
<td>Control⁴</td>
<td>78.0 ± 18.1</td>
<td>80.6 ± 17.5</td>
</tr>
<tr>
<td>fMLP</td>
<td>104.0 ± 42.5</td>
<td>104.5 ± 30.1</td>
</tr>
<tr>
<td>Control¹</td>
<td>65.9 ± 9.0</td>
<td>144.4 ± 23.4⁶</td>
</tr>
<tr>
<td>LPS</td>
<td>57.9 ± 6.2</td>
<td>92.5 ± 11.7⁶</td>
</tr>
<tr>
<td>TNF-α</td>
<td>75.7 ± 12.6</td>
<td>195.0 ± 25.4⁶</td>
</tr>
</tbody>
</table>

* Ratio of the mean of normal vessels to that of tumor vessels after adjustment for the pseudo shear rate.

⁴ fMLP experiments.

⁵ Significantly >1 by the likelihood ratio test (P < 0.05), which means that the tested parameter (i.e., Frolling or Dadhering) of normal vessels is significantly greater than that of tumor vessels.

⁶ LPS and TNF-α experiments.

⁷ Significantly increased from normal vessels by Student’s t test (P < 0.05).

Fig. 3. Comparison of leukocyte rolling and adherence frequency between normal and tumor tissues under LPS or TNF-α stimulated conditions and their corresponding control condition. The average flux of rolling leukocytes (Frolling) (A) and the average density of adhering leukocytes (Dadhering) (B) were compared between normal and tumor tissues, under control, LPS, or TNF-α stimulated conditions. Numbers in parentheses, number of vessels examined. *, a statistically significant difference after each parameter was adjusted for γ₅, between tumor and normal tissues under the same experimental condition (/> 0.05). Bars, SEM.
Endothelial cells.

We noticed that the interaction between leukocytes and endothelial cells appeared normal in the normal tissues surrounding tumors. Since the tumor and the surrounding normal tissues were perfused by the same leukocytes, the diminished L/E interaction in tumor tissues is most likely attributed to altered properties of endothelial cells in the tumor. Many endothelial surface adhesion molecules involved in L/E interaction have been identified. Our observations that L/E interaction was reduced under various stimulated conditions provide certain clues as to which adhesion molecules might be improperly expressed. For example, it is known that fMLP can up-regulate the expression of CD11/CD18 molecules on leukocytes and promote binding to their receptors on endothelial cells, i.e., ICAM-1 and ICAM-2 (5). Our finding of a reduced L/E interaction in tumor during fMLP stimulation suggests that the basal expression of ICAM-1 and ICAM-2 on tumor endothelial cells is probably reduced. Treating endothelial cells with LPS or TNF-α for 4 h is known to stimulate the expression of endothelial leukocyte adhesion molecule 1 (ELAM-1 or E-selectin) and the endothelial ligand for leukocyte adhesion molecule 1 (L-selectin, an adhesion molecule on leukocytes) (5, 10). Thus our finding of lower L/E interaction after LPS or TNF-α treatment suggests inadequate expression of the above two adhesion molecules on tumor endothelial cells. Lack of ELAM-1 expression in certain tumor models has been observed previously (11). It remains uncertain whether the only other known adhesion molecule on endothelial cells, i.e., GMP-140 (P-selectin), is also expressed improperly in tumors. It is known that GMP-140 is colocalized with vWF on Weibel-Palade bodies of endothelial cells, and that its expression results from the fusion of vWF-containing granules with endothelial membrane (12, 13). Bradykinin has been shown to stimulate vWF release from endothelial cells (14), thus is also likely to stimulate the expression of GMP-140. Interestingly, we have observed previously that L/E interaction during bradykinin stimulation was much less prominent in tumor than in normal tissues (4). Therefore, it would not be very surprising if GMP-140 is also improperly expressed on tumor endothelial cells.

Although we have demonstrated that leukocytes interact with endothelial cells less in tumor than in normal tissues, we could not exclude the possibility that this phenomenon is specific to the tumor model used in our study. However, a similar differential regarding leukocyte adherence between normal and tumor tissues has been observed under control conditions in a rabbit ear chamber bearing VX2 carcinoma (15), and our preliminary investigation suggests that L/E interaction may also be reduced in a fibrosarcoma model (data not shown). In addition, we do not know the relative contributions of the various types of leukocytes to the observed differences in leukocyte-endothelium interaction. It has been reported that lymphokine-activated killer cells were localized preferentially in tumor microcirculation (15). This apparent discrepancy could be due to the difference in the tumor model used in that study from that of ours, or, alternatively, due to retained ability of endothelial cells in tumor to interact with this specific class of leukocytes.

If the lack of leukocyte-endothelium interaction is common in different tumor types, this phenomenon may have important implications to different aspects of cancer therapy. For instance, adoptive immunotherapy (3) would be most likely to succeed in tumors in which the leukocytes used for the therapy would adhere to and migrate through endothelial cells. It has been reported, in in vitro systems, that irradiation of endothelial cells generated a lipid neutrophil chemoattractant (16) and promoted neutrophil adhesion (17). Therefore, in radiation therapy, higher endothelial adhesive capacities for leukocytes in normal vasculature could lead to a higher level of leukocyte adhesion to these endothelial cells, resulting in more leukocyte-related injuries in blood vessels of normal tissues relative to that in tumors. Differential leukocyte adhesion to endothelial cells could also alter the distribution of vascular resistance (1) and thus the distribution of blood flow, between normal and tumor tissues.

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