Abstract. Background/aim: Combretastatin A-4 phosphate (CA4P) is a vascular-disrupting agent which affects the level of circulating neutrophils. Since tumor-associated macrophages and neutrophils may collaborate, we compared the effect of CA4P treatment on monocytes/macrophages and neutrophils.

Material and Methods: CDF1 mice with a C3H mammary carcinoma foot tumor were injected intraperitoneally with CA4P. Blood samples were taken and tumors excised at various time-points after treatment. Circulating monocytes and granulocytes were detected by flow cytometry and the tumor levels of these cell types was estimated immunohistochemically.

Results: CA4P induced similar oscillating effects on the level of circulating monocytes and of neutrophils, with an initial decrease followed by an increase and a return to control levels at 6-h and 24-h, respectively. In tumors, only the macrophage level decreased significantly after treatment. Conclusion: CA4P induced similar changes in the level of circulating monocytes and neutrophils, but only affected the fraction of macrophages significantly.

Combretastatin A-4 phosphate (CA4P) is a vascular-disrupting agent (VDA) that selectively targets the tumor vasculature (1, 2). Pre-clinical studies have demonstrated that CA4P can reduce tumor perfusion, enhance tumor necrosis, and inhibit tumor growth (1, 3, 4) although the exact causes of the anti-tumor effects are not fully understood (2).

The anti-tumor effect of the drug on its own is limited because a rim of viable tumor cells remains at the tumor periphery (1). However, preclinical studies have shown significant improvements in tumor response can be obtained if CA4P is combined with more conventional therapies, such as radiation and chemotherapy (3–7). CA4P is now the leading VDA in clinical development (8–11).

Macrophages and neutrophils are two types of leukocytes that are a natural component of tumors (12). Both cell types possess the ability to be either pro- or anti-tumorigenic (13, 14) and are theoretically capable of mediating damage to vessels (13, 15), as well as inducing angiogenesis (13, 16, 17). Matrix metalloproteinase 9 (MMP9) is involved in angiogenesis, tumor growth, and metastasis [reviewed in (18)], and can be produced by both tumor-associated macrophages and neutrophils (19). Some tumor-associated macrophages possess the ability to limit neutrophil infiltration into tumors (20). But in the absence of macrophages, MMP9-positive neutrophils take over the support of tumor angiogenesis (20). Hence, there appears to be some form of collaboration between macrophages and neutrophils. Thus, we hypothesized that macrophages and neutrophils, or the relationship between these cells, may be affected by CA4P administration and we investigated such a potential relationship in a murine tumor model.

Material and Methods

Animal and tumor models. Male CDF1/Bom mice were obtained from Taconic Laboratories (Ry, Denmark). When mice reached 10 to 14 weeks old they were implanted with a C3H mammary carcinoma in the right rear foot. This tumor model is a standard model used in our laboratory for testing VDA (21). The derivation and maintenance has previously been described (22). Tumor-bearing mice were used for experimentation 10–20 days after inoculation, when tumors reached a size of about 150–250 mm3. Tumor volume was calculated from the formula D1×D2×D3×π/6, where the D values represent the three orthogonal diameters. All animal experiments were conducted according to the animal welfare policy of Aarhus University (http://dyrefaciliteter.au.dk) with the Danish Animal Experiments Inspectorate’s approval (J.nr.2010/561-1919, C5).

Drug. Combretastatin A-4 phosphate (CA4P) was supplied by OXIGENE, Inc. (South San Francisco, CA, USA). The drug was prepared fresh before each experiment, by dissolving in saline, and kept cold and protected from light until used. It was injected...
intrapitoneally (i.p.) at a constant volume of 0.02 ml/g bodyweight to give a final dose of 25 mg/kg.

**Blood samples and tumor excision.** Mice in the treatment groups were i.p injected with CA4P and at 1, 3, 6, 24, 48, 72, 96, 120, or 144 hours after injection, a blood sample was withdrawn from the animals sub-orbital sinus. Control animals received an i.p. injection of saline and a blood sample was withdrawn 1 hour after injection. To avoid repetitive blood sampling from animals with small blood volumes, only one blood sample was withdrawn from the

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**Table I. In vitro effect of 2-h incubation of whole blood in the presence of combretastatin A-4 phosphate (CA4P) or combretastatin A-4 (CA4) (both at 10 μM), or saline on the percentage of apoptotic (annexin V+) and dead (7-aminoactinomycin D (7AAD+) monocytes and neutrophils, as determined using flow cytometry.**

<table>
<thead>
<tr>
<th>Monocytes (%)</th>
<th>Neutrophils (%)</th>
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<tbody>
<tr>
<td></td>
<td>Annexin V+</td>
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<tr>
<td>Control</td>
<td>11.7±0.1</td>
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<tr>
<td>CA4P</td>
<td>17.0±1.2</td>
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<tr>
<td>CA4</td>
<td>16.8±1.3</td>
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Data are presented as median±SEM, (n=2-3).

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**Figure 1.** A: Relative monocyte (open symbols) and neutrophil (closed symbols) densities in peripheral blood as a function of hours after treatment with 25 mg/kg combretastatin A-4 phosphate (CA4P). Data are presented as median values with error bars representing the 25th and 75th percentiles; n=5-15. B: Correlation between the relative monocyte density and neutrophil density; r=0.790, p<0.001. C, D: Representative dot plots of forward and side scatter of peripheral blood from a control mouse (C) and a blood sample taken 1 hour after CA4P treatment (D). The circular gate encircles the granulocytes; the squared gate encircles the fluorescent TruCOUNT beads used to calculate the absolute number of cells in a sample. E,F: Representative dot plots of CD14 versus Gr-1 of peripheral blood from a control mouse (E) and a blood sample taken 1 hour after CA4P treatment (F); the gates encircle the monocytes.
sub-orbital sinus from each mouse. The blood samples were drawn directly into dry EDTA-tubes (Sarstedt, Nümbrecht, Germany) and were analyzed by flow cytometry as described below. After the blood samples were taken, mice were killed by cervical dislocation and the tumors were excised and fixed in 4% formaldehyde and used for immunohistochemistry (described below).

CA4P effect on granulocyte and monocytes density in peripheral blood. Collected blood samples were transferred to TRuCOUNT tubes (BD Bioscience, Broendby, Denmark), used for absolute count of leucocytes or falcion tubes (BD Biosciences), depending on the experiment. Both tube types were incubated for 15 minutes at room temperature with anti-granulocyte receptor-1 (Gr-1) (Cedarlane, Ontario, Canada) and anti-CD14 (Nordic BioSite, Copenhagen, Denmark) antibodies. Red blood cells were lysed using Stock solution ×10 (Ampliqon Bioreagents and Molecular Diagnostics, Skovlunde, Denmark) and the samples were analyzed using flow cytometry (FACS Calibur or FACSContour™ II; BD Biosciences) and FlowJo (version 9.3.1; Tree Star Inc., Ashland, OR, USA). The anti-Gr-1 antibody binds to Ly-6G, expressed by neutrophils, and to Ly-6C, expressed by neutrophils, dendritic cells, and subpopulations of lymphocytes and monocytes (23). Thus granulocytes were identified based on localization in a dot-plot of forward and side scatter and by being Gr-1 positive. Anti-CD14 binds to CD14, which is strongly expressed on monocytes and granulocytes. Hence, monocytes were defined by being CD14-positive and Gr-1<sup>+</sup> and by their localization in a dot-plot of forward side scatter in combination with localization in a dot plot of CD14 versus Gr-1 scatter. A TRuCOUNT tube contains a known number of fluorescent beads and the cell density was calculated by dividing the number of positive cellular events (monocytes or granulocytes) by the number of bead events, and then multiplying by the TruCOUNT bead count. In this way, the number of monocytes or granulocytes per volume of blood could be calculated. As a positive control, blood samples from mice killed by cervical dislocation were used. Hence, the estimated cell density was lower than the true cell density, but it was feasible to compare the results obtained with the two methods described above.

Estimation of neutrophil or macrophage area fraction in histological sections. Tumors fixed in 4% formaldehyde (described above) were embedded in paraffin. For each tumor, two sections of 2-3 μm thickness, 200 μm apart, were produced. Endogenous peroxidases were blocked with H<sub>2</sub>O<sub>2</sub>, followed by thorough rinsing in a buffer containing phosphate buffered saline (PBS) and Tris-Ethylenediaminotetraacetic acid-glycose (TEG) buffer, pH 9.0. The sections were then transferred to a LabVision Autostainer 480 (Labvision, Fremont, CA, USA) and for visualization of macrophages, sections were incubated overnight with rat anti-mouse F4/80 primary antibody (Nordic BioSite) followed by 30 min. incubation with rabbit-anti-rat secondary antibody both (Nordic BioSite). For visualization of neutrophils, sections were incubated overnight with a polyclonal rabbit antibody staining myeloperoxidase (Nordic BioSite). The primary and secondary antibody was detected by incubating sections for 30 minutes with anti-rabbit IgG-horseradish peroxidase-conjugated polymers (DakoCytomation). Finally, sections were counterstained with Mayer’s hematoxylin and sections were scanned using a NanoZoomer 2.0-HT (Hamamatsu Photonics K.K., Hamamatsu City, Japan). To estimate the degree of neutrophil and macrophage infiltration, we estimated the area fractions of positively stained profiles in the tumors by point-counting at x20 magnification. The area fractions were estimated using a counting frame (240×240 μm, consisting of 100 equidistantly spaced points). This counting frame was sampled in a systematic random fashion throughout the tumor section and both vital and necrotic areas in the tumor tissue were counted. Points hitting artifacts or cells from normal non-neoplastic connective-tissue were ignored, as were the macrophage and neutrophil profiles located in these areas. A neutrophil cell profile was defined as a cell with myeloperoxidase-positive granules located in close proximity to a nucleus. The area fraction was calculated by dividing the number of points hitting a neutrophil or macrophage by the number of points hitting tumor tissue.

Statistics. Macrophage and neutrophil densities in peripheral blood and in tumors and the percentage of dead and apoptotic monocytes in the in vitro experiment were compared using two-tailed Student’s <i>t</i>-test for comparison of two groups, and one-way ANOVA or Mann–Whitney rank sum tests were used for comparison of more than two groups. The correlation between macrophage and neutrophil densities in peripheral blood were compared using Pearson’s correlation. For all tests, a <i>p</i>-value of less than 0.05 was considered statistically significant.

Results

CA4P mediated changes in monocytes and neutrophil concentrations in peripheral blood. Figure 1A illustrates the change in monocyte and granulocyte densities in peripheral blood as a function of hours after treatment with CA4P (25 mg/kg). This drug mediated an oscillating effect on the monocyte density [median (95% confidence interval (CI)] with a relative decrease to 0.61 [0.46-0.74] at 1 h after drug administration (<i>p</i>=0.012) followed by an increase to 1.51 (1.05-1.97) at 3 h after treatment (<i>p</i>=0.031 compared to control). At later time-points (6-48 h), the monocyte density returned to the level of that of the control mice, but at 72 h after injection, it had increased to 3.26 (2.64-3.87). This increase lasted until at least 144 h after injection. The level of granulocytes in peripheral blood similarly changed, except that it returned to the level of the controls 24 h after CA4P administration. At time-points later than 72 h, the monocyte density was higher than the granulocyte density albeit this difference was only significant at 96 and 144 h (<i>p</i>=0.003 and <i>p</i>=0.032, respectively). As shown in Figure 1B, the macrophage
Figure 2. Histologically-estimated area fraction of macrophages (A) and neutrophils (B) in C3H mammary carcinomas in mice as a function of time after treatment with combretastatin A-4 phosphate (CA4P, 25 mg/kg). Data are presented as the mean±SEM (n=3). * significantly different from controls. Representative histological sections from control tumors (C and D) and tumors excised 1 h after CA4P treatment (E and F), showing the F4/80-positive macrophages in brown in C and E, and the myeloperoxidative neutrophils in brown in D and F.
density, measured for each mouse at various time-points, consistently correlated with the neutrophil density (r=0.790, p<0.001).

In vitro toxicity assay. To investigate whether CA4P, or the active drug CA4, were toxic to monocytes, we incubated whole blood in the presence of saline (control), CA4P, or CA4. After 2 h of incubation, the percentage of 7AAD-positive (dead), annexin V-positive (apoptotic) and the double-positive monocytes were determined using flow cytometry. As shown in Table I, neither CA4P nor CA4 changed the percentage of apoptotic or dead monocytes compared to control cells.

CA4P mediated changes in tumor infiltration of macrophages and neutrophils. Figure 2 illustrates the estimated area fraction of macrophages and neutrophils in tumor sections from control mice and from tumors excised at different time-points after CA4P treatment. In controls, the estimated area fraction of macrophages was 5.5±0.5% and 2.9±0.6% for neutrophils. CA4P significantly reduced the macrophage area fraction to 1.9±0.5% (p=0.002), 3.1±0.6% (p=0.009), and 1.23±0.4% (p=0.001) at 1, 3, and 6 hours after treatment, respectively. In contrast, we found no changes in the area fraction of neutrophils at any of the investigated time-points (p=0.718).

Discussion

In the present study we determined the effect of CA4P treatment on the monocyte and neutrophil densities in peripheral blood and on the levels of tumor-associated neutrophils and macrophages. CA4P mediated a uniform oscillating change in monocytes and neutrophils in peripheral blood. In an earlier study, we compared the neutrophil levels in non-tumor-bearing and tumor-bearing animals following treatment with CA4P (24). The effects observed during the first 6 h were similar, but the increase from 72 h was only seen in the tumor-bearing mice. This led us to suggest that while the early (within the first 6 h) changes were caused by a non-specific recruitment of neutrophils in CA4P-mediated vascular effects in both normal and tumor tissue, the latter (≥72 h) effects were simply the result of mice having a tumor.

Because the variations in monocyte and neutrophil densities in the study presented herein were similar, we suggest that the early (within the first 6 h) changes in monocyte level were caused by non-specific recruitment of these cells to the CA4P-mediated damage. As to why the neutrophil density is increased for a longer period than the monocyte level, we suggest that a higher number of neutrophils than monocytes are released from the bone marrow.

CA4P treatment mediated a significant decrease in the estimated area fraction of tumor-associated macrophages at all three time points investigated compared to the control (Figure 2). In contrast, no changes were found in the estimated neutrophil area fraction. This result was surprising since we had expected either no effect or an increase in tumor-infiltrating macrophages as a result of the CA4P-mediated vascular damage. The decreased detection of macrophages can be caused by a diminished number of these cells in the tumor tissue. However, because we observed the decrease as early as 1 h after CA4P administration, we find it more likely that there is another explanation for this observation. F4/80 is constitutively expressed on most resident tissue macrophages. However, expression of the antigen increases as cells mature in culture (25). It is, therefore, possible that monocytes had entered the tumor at the investigated time-points, but F4/80 expression on these cells may have been lower than on mature macrophages. This could explain the stable level of macrophages, but not the decreased detection of F4/80-positive cells. Another explanation could involve the fact that F4/80 has been shown to be down-regulated on macrophages in response to inflammation (26). Since CA4P mediates vascular damage, it is likely that it also initiates an inflammatory response which may lead to down-regulation of F4/80 (26). A previous study showed that CA4P treatment significantly increases tumor infiltration by both Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE2) - expressing F4/80-positive macrophages and Gr-1-positive neutrophils 24 h after treatment (27), a time-point that we did not investigate. Furthermore, that previous study showed that inhibiting recruitment of TIE2-expressing macrophages increased the efficacy of CA4P treatment, by reducing tumor growth time, and stimulated an increase in neutrophil infiltration into tumors. These data, in addition to ours, indicate that there may be a co-regulation of macrophages and neutrophils following CA4P treatment.

Conflicts of Interest

None to declare.

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References


