ANNALS OF
Cancer Research and Therapy
Copyright © 2003 By
PJD PUBLICATIONS LTD.
Biomedical Publisher to the World
P. O. BOX 966, WESTBURY, NY 11590

**TUMOR MICROCIRCULATION OBSERVED BY A CHARGE-COUPLED
DEVICE MICROSCOPY AND ANTITUMOR EFFECT BY STRANGULATION**

•
Suguru Sawada, Hajime Hirose, Yoshio Mori, Hisato Takagi and Hisashi Iwata

Department of Advanced Surgery, Division of Organ Pathobiology,
Gifu University School of Medicine
40 Tsukasa-machi, Gifu, 500-8705 Japan

Key Words: AH130 cells, anti-tumor effect, charge-coupled device microscope (CCD),
ischemia-reperfusion, microcirculation, strangulation, tumor volume.

Abstract

The purpose of this study was to examine the antitumor effect of strangulation to cease blood flow on tumor tissue. AH130 cells were implanted in the mesentery near the ileocecal portion of rats 10 days previously. A recovery rate of blood flow was observed in the tumor tissue and normal tissue (cecal wall) after reperfusion with a charge-coupled device microscope (CCD). Five days after reperfusion, the tumor volume ratio was measured. On the death or sacrifice of the animals, the tumor necrotic area ratio was examined. The vascular morphology and the changes of a recovery rate of blood flow after reperfusion differed from the tumor tissue and the normal tissue by CCD observation. In the 15-min and 30-min groups, the tumor vessels underwent little or no destruction, with only a few showing a cessation of blood flow. However, the 60-min and 90-min groups showed a cessation of blood flow and hemorrhage due to vascular destruction in many

Corresponding author. ; Suguru Sawada, MD, Ph.D., TEL.: +81-58-267-2619; FAX:
+81-58-267-2955; E-mail address: sawada-s@isis.ocn.ne.jp. Manuscript received
03/04/03 and accepted 04/01/03.

vessels, and few vessels showed intact blood flow. In 60 and 90-minutes strangulated groups, a recovery rate of blood flow after 60 minutes was lower in tumor tissue than in the normal tissue. The tumor volume ratio decreased more in the 60 and 90-minutes strangulated groups than in the non-strangulated group and the tumor necrotic area increased more in all strangulated groups than in the non-strangulated group. These findings suggest that strangulation for ischemia for 60-90 minutes injures the tumor tissue and shows the antitumor effect.

Introduction

Denekamp, Hill *et al.* (1983) have shown that vascular occlusion results in marked tumor regression, delayed growth and long term tumor control. Their study has recently led to the development of tumor therapy focusing on tumor vessels. Nihei, Suga *et al.* (1999) and Yoshikawa, Kokura *et al.* (1994) developed these therapies using ischemia-reperfusion with transient embolic agents such as degradable starch microspheres and combretastatin.

It seems to be important for these therapies to observe the changes of blood flow in the tumor tissue. But Nihei, Suga *et al.* (1999) did not observe the blood flow. Yoshikawa, Kokura *et al.* (1994) observed the blood flow by Doppler flow meter. Suzuki, Yanagi *et al.* (1996) reported the blood flow observation in the tumor tissue using a confocal laser scanning microscope, and Nasu, Kimura *et al.* (1999) using a charge-coupled device microscope (CCD) without intervention.

The therapies using ischemia-reperfusion with transient embolic agents such as degradable starch microspheres and combretastatin avail clinically. But it is not clear whether these therapies are effective for antitumor therapy and do no damage to the normal tissue.

The purpose of this experiment was to investigate whether the strangulation procedure exerted antitumor effects controlling the growth of a tumor. Using CCD, we examined changes in the microcirculation of the tumor and the normal tissue implanted in

the mesentery during ischemia-reperfusion by strangulation to analyze the differences in microcirculation and the pathologic changes of tumor tissue.

Materials and Methods

All investigations adhered to the Guide for the Care and Use of Laboratory Animals of the National Research Council, U.S. Department of Health and Human Services and National Institutes of Health. Six-week-old, male Donryu rats (Japan SLC, Hamamatsu) weighing 130, 150 g were used in this experiment.

First, AH130, rat ascetic hepatocellular carcinoma cells, provided by Tsukuba ARL, 1×10^6 cells/ml was suspended in RPMI-1640. The rats underwent a laparotomy under ether anesthesia, and 0.1 ml of the cell suspension was implanted into the mesentery in the ileocecal region.

On the tenth day after implantation, a re-laparotomy was performed under anesthesia with 30 mg / kg of pentobarbital sodium i.p. The tumor diameter was measured, and the tumor volume (cm^3) = long diameter $\times$ (short diameter) $^2 \times 0.5$, was calculated according to the method described by Carlsson, Gullberg *et al.* (1983).

According to the method of Park, Haglmd *et al.* (1990), strangulation with a two mm-diameter rubber tube was applied to the implanted tumor together with an adjacent approximately 5-cm-long ileocecal segment of the bowel to cease blood flow at normal temperature. Heparin was not used. The rats were divided into a 15-min group (n=8), 30-min group (n=8), 60-min group (n=10), and 90-min group (n=8) according to the duration of strangulation. The group without strangulation served as the control (C) group (n=8). Before and during strangulation, and immediately (0 minute), 15, 30, 45, and 60 minutes after reperfusion, the serosal surface of the strangulated cecum and the tumor surface were observed at 5 randomly selected points under CCD (Digital

Microscope VH-6200, KEYENCE CORP., Osaka) with a 500 x lens according to the methods of Suzuki, Yanagi *et al.* (1996) and Furuzawa, Hirose *et al.* (1996). The images of blood flow in the vessels were recorded with a digital videocassette recorder (WV-D10000, SONY CORP., Tokyo). Complete cessation of blood flow during strangulation was confirmed with CCD (Sawada, 2003).

Blood flow in the vessels was analyzed as follows. First, only vessels 10-30 μm in diameter in a 0.15 mm^2 area of the image input with the videocassette recorder were observed according to the method of Suzuki, Yanagi *et al.* (1996). The total number of vessels and the number of vessels showing blood flow (blood flow-positive vessels) were counted in a randomly selected area according to the method of VanBavel and Spaan (1992). The blood flow-positive ratio was calculated by dividing the total number of blood flow-positive vessels by the total number of vessels: blood flow-positive ratio = total number of blood flow-positive vessels at 5 points / total number of blood vessels at 5 points (Sawada, 2003).

The blood flow-positive ratios at 0-60 minutes after reperfusion were compared with those before strangulation: recovery rate of blood flow = (blood flow positive ratio at each post-reperfusion time / pre-strangulated blood flow-positive ratio) x100. The recovery rate of the strangulated bowel (normal tissue) and tumor blood flow was described as NR (Normal tissue Recovery rate) and TR (Tumor tissue Recovery rate), respectively. Then, time-dependent changes in NR and TR were compared in each strangulated group. The abdomen was closed at 60 minutes after reperfusion.

On the fifth day after reperfusion, the changes of rates of tumor volume (TVR) was calculated according to the method of Wang, Persson *et al.* (1994) as TVR = tumor volume at 5 days after reperfusion / tumor volume before strangulation after the tumor

diameter was measured. The tumor volume in the C group and the pre-strangulated tumor volume in each strangulated group were compared only in rats surviving for more than 15 days after tumor implantation (C group, n=7; 15-min group, n=6; 30-min group, n=7; 60-min group, n=10; and 90-min group, n=5).

Subsequently, the abdomen was closed again, and rats were kept alive. They died naturally after implantation in the tumor before 30th day. The tumor was resected, and then, histological sections were stained with hematoxylin and eosin. First, the tumor was cut through the maximum diameter, and the section for their histology was prepared. The sections were photographed on 35 mm slides using a stereoscopic microscope (x8.5), and the images were input into a Power Macintosh G3 (Apple Computer, Inc., USA) using Adobe Photoshop 4.0E. Changes in tumor tissue were evaluated in terms of the tumor necrotic area ratio according to the method of Sakamoto, Hirose *et al.* (2000) and Niwano, Arii *et al.* (1998). These images of the tumor tissue and necrotic areas were divided by the threshold values using NIH Image V1.6, and the ratio of necrotic area to the total area of a tumor section, designated the tumor necrotic area ratio, was calculated (Sawada, 2003).

Statistical analysis was performed as follows. Repeated ANOVA compared the time course of NR with TR, t-test compared NR with TR in 60 minutes after reperfusion and the Bonferroni-Dunn procedure compared TVR, tumor necrotic area ratio and NR and TR in 60 minutes after reperfusion among the groups. P values less than 5% were considered significant.

Results

Observation of blood flow with CCD

Tumor vessels were formed as the network from the mesentery to tumor surface.

The vascular morphology of the normal tissue (cecal serosa) was lattice-like and consisted of vessels with an even diameter, with blood flowing in all of them. In contrast, the tumor vessels varied in length and diameter, were ramified into a network, and showed poor blood flow in some areas even before strangulation. The number of blood flow positive vessels, among vessels in which blood flow once ceased, increased with time after reperfusion in both normal and tumor tissue. Normal tissue vessels did not show any vascular destruction morphologically.

Many of them showed stagnant blood flow with retention of erythrocytes in the lumen, or collapsed by ischemia with a resultant reduction of their diameter. The number of vessels stagnated blood flow and collapsed tended to increase with the duration of strangulation. On the other hand, in the 15-min and 30-min groups, the tumor vessels underwent little or no destruction, with only a few showing a cessation of blood flow. However, the 60-min and 90-min groups showed a cessation of blood flow and hemorrhage due to vascular destruction in many vessels, and few vessels showed intact blood flow (Figure 1a-d).

Figures 2(a), 2(b), 2(c) and 2(d) present the per cent recovery rates at 15, 30, 60 and 90 minutes respectively. The results in Figure 3 compare the recovery rates of NR and TR at different time schedules. Figure 4 shows the tumor volume ratio of the control, 15 min, 30 min, 60 min and 90 min groups. Figure 5 presents the necrotic area ratio for the control and the same stated time intervals.

The analysis of the time course of TR and NR showed that NR was significantly higher than TR in all strangulated groups ($p<0.01$, $p=0.01$, $p<0.01$, and $p<0.01$ for the 15-min, 30-min, 60-min, and 90-min groups, respectively) (Figure 2a-d).

Figure 1 : Blood vessels of the tumor and the normal tissue before strangulation and after reperfusion investigated by the CCD microscope (x500). (a): (TOP) Normal vessels before strangulation (b): (BOTTOM) Tumor vessels before strangulation.

Figure 1: (continued): Blood vessels of the tumor and the normal tissue before strangulation and after reperfusion investigated by the CCD microscope (x500). (c): (TOP) Normal vessels after reperfusion (d): (BOTTOM) Tumor vessels after reperfusion

Figure 2 : The comparison of TR and NR after strangulation for 15 minutes (TOP - a), 30 minutes (BOTTOM - b). NR was significantly higher than TR in all strangulated groups (a): $p < 0.01$, (b): $p = 0.01$ respectively.

Figure 2 : (continued) The comparison of TR and NR after strangulation for 60 minutes (TOP - c) and 90 minutes (BOTTOM - d), respectively. NR was significantly higher than TR in all strangulated groups (c): $p < 0.01$, and (d): $p < 0.01$.

NR in 60 minutes after reperfusion was 100%, $99.4 \pm 1.7\%$, $79.2 \pm 14.2\%$ and $74.2 \pm 12.0\%$ and TR in 60 minutes after reperfusion was $98.3 \pm 3.5\%$, $93.2 \pm 10.0\%$, $46.7 \pm 11.1\%$ and $34.3 \pm 11.2\%$ in 15-min group, 30-min group, 60-min group and 90-min group, respectively. There were no significant differences between NR and TR in 15 and 30-min groups. However, there were significant differences between NR and TR in 60 and 90-min groups ($p < 0.01$, respectively) (Figure 3).

Figure 3 : The comparison of NR and TR in 60 minutes after reperfusion for each strangulated groups. There were no significant differences between NR and TR in 15 and 30-min groups (*). However there were significant differences between NR and TR in 60 and 90-min groups (†: $p < 0.01$, respectively)

On comparing NR in 60 minutes after reperfusion with each four group, there were significant differences between 15 VS 60-min, 15 VS 90-min, 30 VS 60-min and 30 VS 90-min groups ($p < 0.01$, respectively). In the comparison of TR in 60 minutes after

Figure 4 : (TOP) Tumor volume ratio (TVR) comparing the C group with each strangulated group, respectively. The 30-min group showed a significantly higher TVR ($p < 0.01$), whereas the 60-min and 90-min groups showed a significantly lower TVR ($p < 0.05$ for the 60 and 90 min groups, respectively).

Figure 5 : (BOTTOM) Tumor necrotic area ratio comparing the C group with each strangulated group, respectively. Thus, all strangulated groups had a significantly higher tumor necrotic area ratio than that the C group ($p < 0.01$).

reperfusion with each four group, there were significant differences between 15 VS 60-min(†), 15 VS 90-min(†), 30 VS 60-min(†), 30 VS 90-min(†) and 60 VS 90-min groups(‡) (†; $p<0.01$, ‡; $p<0.05$, respectively).

Changes in tumor volume

The pre-strangulated tumor volumes (cm^3) did not significantly differ among the groups. TVR was as follows: the C group, 1.94 ± 0.99 ; the 15-min group, 3.14 ± 1.58 ; the 30-min group, 4.26 ± 2.16 ; the 60-min group, 0.54 ± 0.34 ; and the 90-min group, 0.61 ± 0.24 . Compared with the C group, the 30-min group showed a significantly higher TVR ($p<0.01$), whereas the 60-min and 90-min groups showed a significantly lower TVR ($p<0.05$ for the 60 and 90 min groups, respectively) (Figure 4).

Changes in the tumor tissue after strangulation

In all tumors, most of the tumor vessels existed at the surface of the tumor and the necrotic area existed at the central of the tumor. The differences between the viable tumor tissue and necrotic tissue were clear. The tumor necrotic area ratios reflecting injury to tumor tissue were as follows: the C group, 11.2 ± 6.6 ; the 15-min group, 31.4 ± 3.2 ; the 30-min group, 32.8 ± 13.7 ; the 60-min group, 37.0 ± 15.2 ; the 90-min group, 48.1 ± 5.4 . Thus, all strangulated groups had a significantly higher tumor necrotic area ratio than that the C group ($p<0.01$) (Figure 5).

Discussion

It has been reported that ischemia-reperfusion causes profound injury to tumor tissue but less to the normal tissue (Denekamp, Hill *et al.*, 1983). Hori, Saito *et al.* (1999) reported that the blood flow in the normal tissues generally recovers to its original level but that in the tumor blood flow never recovers in many regions in the study with combretastatin. The purpose of our study was to elucidate antitumor effect with

ischemia-reperfusion by strangulation of the tumor tissue through the observation of microcirculation with CCD microscope and morphological changes. There have been reports of blood flow observation in the tumor tissue using a confocal laser scanning microscope (Suzuki, Yanagi *et al.*, 1996) or CCD without intervention (Nasu, Kimura *et al.*, 1999) and with administration of combretastatin (Hori, Saito *et al.*, 1999). We observed its microcirculatory changes with CCD microscope before strangulation and after its release. The strangulation procedure is to squeeze the bowel and tumors on the mesentery to make an ischemic state in the tumor tissue. This is the first report of the procedure that is designed to control the growth rate of the unresectable tumors on the mesentery by reducing its blood flow and which was observed by CCD.

In our study, we developed a rat model with tumor cells injected in the mesentery. Our model had the advantage that the tumor diameter could be measured repeatedly to assess the antitumor, as the implant of a tumor was achieved by simple injection of the tumor cell suspension into the mesentery. AH130, rat ascetic hepatocellular carcinoma cell that we used, is easy to judge the antitumor effect morphologically and to observe the tumor blood flow because the tumor formed by AH130 was hypervascular. Two reasons why re-laparotomy was performed on the tenth day after implantation were that most suitable tumor volume to study became and peritoneal dissemination did not exist on that time.

Denekamp, Hill *et al.* (1983) have reported that 4 to 8 hours clamping of blood vessels into the subcutaneous tumor induced significant tumor cell death. Yasumura, Mori *et al.* (2003) reported that all rats died of 90 minutes strangulation of the ileum 20cm in length and survived in 45 minutes strangulation. In our experiment, since strangulation involved a part of the intestine and cecum 5cm in length, the longest strangulation time in

our experiment was 90 minutes. In our experiment, strangulation for 60-90 minutes caused tumor shrinkage or tumor cell death. Strangulation for 60-90 minutes decreased the tumor volume and increased the tumor necrotic area. These findings suggest that strangulation for ischemia for 60-90 minutes injures the tumor tissue and shows the antitumor effect.

Two theories have been proposed to explain the mechanism of tumor shrinkage due to ischemia. One suggests that reduced blood flow due to ischemia-reperfusion directly induced the death of tumor cells (Wang, Persson *et al.*, 1994). According to another theory, peroxide stress resulting from reperfusion injures the endothelial cells of vessels supplying tumor cells, leading to a secondary decrease of blood flow and resultant death of the tumor cells (Parkins, Dennis *et al.*, 1995).

Necrosis and shrinkage of the tumor in our observation suggested that tumor cell death had been caused by ischemia-reperfusion itself or the secondary decrease of blood flow due to injury to the endothelial cells of vessels supplying the blood flow for the tumor.

TVR was calculated 5 days after reperfusion as Wang, Persson *et al.* (1994) reported. Also two mechanisms, which significant increases in TVR was observed in 30-min and in tumor necrotic area ratio was observed in 15 and 30-min, were not defined yet in this experiment.

Denekamp (1991) has reported that tumor cells and tumor vascular endothelial cells synthesize DNA more actively compared with normal tissue cells and endothelial cells. Thus, both tumor cells and tumor vascular endothelial cells are presumably less resistant to ischemia due to the reduction of blood flow.

Algire, Chalkley *et al.* (1945) have reported that unlike vessels in normal tissue, tumor vessels are characterized morphologically by a sinusoid-like structure without

distinct arteries and veins. Suzuki, Yanagi *et al.* (1996) have reported that the vessels of the tumor disseminated in the mesentery show a more complex network than those of normal tissue, and have an uneven diameter on confocal laser scanning microscope observation. The blood flow in tumor vessels is hemodynamically slow and sometimes becomes stagnant (Suzuki, Yanagi *et al.*, 1996 and Nasu, Kimura *et al.*, 1999).

In our experiment, we observed a difference in microcirculation during ischemia-reperfusion by strangulation between the tumor and normal vessels. After reperfusion, it was found that the recovery of blood flow was poorer in the tumor tissue than in the normal tissue in 60 and 90-min groups in the tumor tissue. And TR in 60 minutes after reperfusion was lower in 15 and 30-min groups than those 60 and 90-min groups. Sixty and 90-min groups showed that the tumor vessels were destruction but the normal vessels were not. Thus, we speculate that these results might be due to differences in functions and tolerance of vessels between tumor and normal vessels and we speculate that the tolerance of tumor endothelial cells to strangulation, which might cause ischemia-reperfusion, is less than that of normal endothelial cells.

It has been cleared to differences of the blood flow changes between the tumor tissue and normal tissue and that the recovery of tumor blood flow in the reperfusion after strangulation compared with the normal tissue was slow by CCD observation. In addition, it also has been cleared that strangulation procedure exerted antitumor effects controlling the growth of a tumor.

The ischemia-reperfusion procedure by strangulation against a tumor exerts antitumor effects, although it suspected to cause at least some damages to the normal tissue. It, therefore, seems that we should develop a procedure with little or no damage to the normal tissue in order to use this procedure clinically to control the growth of a tumor.

References

- Algire, G.H., Chalkley, H.W., Legallais, F.Y., Park, H.D. (1945) Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. *J. Nat. Cancer Inst.* 6: 73-85
- Carlsson, G., Gullberg, B., Hafstrom, L. (1983) Estimation of liver tumor volume uses different formulas- an experimental study in rats. *J. Cancer Res. Clin. Oncol.* 105: 20-23
- Denekamp, J. (1991) Anti-vascular cancer therapy: Is it a dream or reality? *Eur. J. Surg. Suppl.* 561: 21-26
- Denekamp, J., Hill, S.A., and Hobson, B. (1983) Vascular occlusion and tumor cell death. *Eur. J. Cancer Clin. Oncol.* 19: 271-275
- Denekamp, J., Hobson, B. (1991) Endothelial-cell proliferation in experimental tumors. *Br. J. Cancer* 46: 711-720
- Furuzawa, Y., Hirose, H., Sasaki, E., Takagi, H., Bando, M. (1996) Distinctive microcirculation of the brain during cerebral perfusion by observation with a CCD microscope. *ASAIO journal* 42: M423-M428
- Hori, K., Saito, S., Nihei, Y., Suzuki, M., Sato, Y. (1999) Antitumor effects due to irreversible stoppage of tumor tissue blood flow: Evaluation of a novel Combretastatin A-4 derivative, AC7700. *Jpn. J. Cancer Res.* 90: 1026-1038
- Nasu, R., Kimura, H., Akai, M., Uda, M., Akagi, K. (1999) Analysis of intermittent blood flow in experimental tumor using a transparent chamber. *Jpn. J. Cancer Chemother.* 26: 667-672
- Nihei, Y., Suga, Y., Morinaga, Y., Ohishi, K., Okano, K., Ohsumi, K., Hatanaka, T., Nakagawa, R., Tsuji, T., Akiyama, Y., Saito, S., Hori, K., Sato, Y. and Tsuruo, T. (1999) A novel combretastatin A-4 derivative, AC-7700, shows marked antitumor activity against advanced solid tumors and orthotopically transplanted tumors. *Jpn. J. Cancer Res.* 90: 1016-1025
- Niwano, M., Arii, S., Mori, A., Ishigami, S., Harada, T., Mise, M., Furutani, M., Fujioka, M., Imamura, M. (1998) Inhibition of tumor growth and microvascular angiogenesis by the potent angiogenesis inhibitor, TNP-470, in rats. *Jpn. J. Surg.* 28: 915-922
- Park, P.O., Haglmd, M.U., Bulkley, G.B., Falt, K. (1990) The sequence of development of intestinal tissue injury after strangulation ischemia and reperfusion. *Surgery* 107: 574-580

- Parkins, C.S., Dennis, M.F., Stratford, M.R.L., Hill, S.A., Chaplin, D.J. (1995) Ischemia reperfusion injury in tumors: The role of oxygen radicals and nitric oxide. *Cancer Res.* 55: 6026-6029
- Sakamoto, K., Hirose, H., Onizuka, A., Hayashi, M., Futamura, N., Kawamura, Y., Ezaki, T. (2000) Quantitative study of changes in intestinal morphology and mucus gel on total parenteral nutrition in rats. *J. Surg. Res.* 94: 99-106
- Sawada, S. (2003) A Charge-coupled Device Microscopic Observation of Tumor Microcirculation during Ischemia-Reperfusion by Strangulation and Antitumor Effect. *Acta. Sch. Med. Univ. Gifu* 51: 120-126
- Suzuki, T., Yanagi, K., Ookawa, K., Hatakeyama, K., Ohshima, N. (1996) Flow visualization of microcirculation in solid tumor tissues: intravital microscopic observation of blood circulation by use of a confocal laser-scanning microscope. *Frontiers Med. Biol. Engng.* 7: 253-263
- VanBavel, E., Spaan, J.A.E. (1992) Branching patterns in the porcine coronary arterial tree. Estimation of flow heterogeneity. *Circulation Research* 71: 1200-1212
- Wang, L.Q., Persson, B.G., Jeppsson, B., Bengmark, S. (1994) Repeated dearterializations of an experimental liver tumor: short- and long- term results. *J. Surg. Res.* 56: 53-59
- Wang, L.Q., Persson, B.G., Stenram, U., Bengmark, S. (1994) Influence of portal branch ligation on the outcome of repeat dearterializations of an experimental liver tumor in the rat. *J. Surg. Oncol.* 55: 229-234
- Yasumura, M., Mori, Y., Takagi, H., Yamada, T., Sakamoto, K.I., Iwata, H., Hirose, H. (2003) Experimental studies to estimate the intestinal viability in a rat strangulated ileus model with CCD microscopy. *Br. J. Surg.* (in press)
- Yoshikawa, T., Kokura, S., Oyamada, H., Iinuma, S., Nishimura, S., Kaneko, T., Naito, Y., Kondo, M. (1994) Antitumor effect of ischemia-reperfusion injury induced by transient embolization. *Cancer Res.* 54: 5033-5035