Suppression of angiogenesis causes a significant delay of repair process in rat thromboembolic cerebral infarction

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Abstract

Enhanced angiogenesis is thought to hold therapeutic potential for ischemic cerebral injuries. However, anti-cancer or immunosuppressive drugs are shown to be able to induce certain degrees of suppression of angiogenesis, and these drugs are also expected to expand the therapeutic window by their neuroprotective intervention through blocking the apoptotic cascade in the penumbra after injury. This experimental study is thus designed to evaluate the effects of suppression of angiogenesis produced by an anti-cancer drug administration in cerebral infarcts of rats.

Cerebral infarction was created in Wistar male rats using a homologous thromboembolic method. The suppression of angiogenesis was induced by intraperitoneal cyclophosphamide (CP) administration two days before the operation. Evaluations were carried out at 8 hours, 1 day, 2 days, 3 days and 5 days in the lesions with or without CP treatment. The number and size of blood vessels were examined statistically. In addition, VEGF (vascular endothelial growth factor) and TGF-beta1 (transforming growth factor-beta1) were studied by immunohistochemistry and western blotting.

In CP-treated rats, the development and maturation of blood vessels were apparently depressed and delayed. At the same time, the appearance of VEGF- and TGF-beta1-immunoreactive cells was delayed, furthermore, the appearance and degree of phagocytosis of macrophages/microglia were also definitely delayed and depressed, resulting in the markedly prolonged persistence of apoptotic cells in the penumbra.

The condition of suppressed angiogenesis with a simultaneous slow process of various cellular response could be beneficial for ischemic brain injuries because it allows us to have a chance to make the therapeutic window be wider and longer. The persistent tissue elements, albeit being necrotic/degenerated, could potentially be recycled to some extent for the subsequent tissue repair.

Introduction

Angiogenesis is known to be induced by tissue hypoxia in cerebral ischemia1. In addition, the onset of angiogenesis has been shown in the border zone of cerebral infarcts2,3. Imaging diagnosis4 and post mortem study5 of stroke patients show that active angiogenesis is more developed in the periphery of the lesions, known as penumbra, and it is correlated with longer survival of the stroke patients. Therefore, the potential therapeutic effects of enhanced angiogenesis have been extensive in cerebral infarction6.
When active proliferation of microvessels occurs in ischemic brain lesions, endothelial cells must have been first activated for proliferation by certain angiogenic factors, which have been demonstrated to be produced and secreted by macrophages, microglia, and astrocytes in the lesions. Among various well known angiogenic factors, both VEGF (vascular endothelial growth factor) and TGF-beta 1 (transforming growth factor-beta 1) have been most extensively studied clinically and experimentally. They have both been demonstrated to be upregulated in stroke patients.

TGF-beta 1 seems to be important for the part it plays in regulating tissue damage and promoting repair. Furthermore, TGF-beta 1 has been suggested to act in neuroprotection. Strong TGF-beta 1 immunoreactivity was found in ischemic neurons, astrocytes, and endothelial cells in both the core area and the penumbra of infarcts. On the other hand, VEGF is clearly induced by hypoxia and is said to be present only in ischemic brains and not in normal brains. VEGF immunoreactivity is demonstrated in ischemic neurons, endothelial cells, and glial cells in the infarcts. VEGF can also inhibit endothelial cell apoptosis and induce chemotaxis in monocytes.

Cerebral ischemia induce tissue death via apoptosis as well as necrosis. Apoptosis may occur in milder forms of ischemic damage, whereas necrosis may predominate in more intense forms of ischemic damage. Apoptotic injury may take time to develop. For example, in one experiment using a photothermobic method of cerebral infarction, apoptotic cells were noted at the border of lesions between 12 hours and 6 days, revealing that neurons at the peripheral areas of lesions eventually succeed to apoptosis. Thus, it is expected that neuroprotective intervention blocking the apoptotic cascade might expand the therapeutic window of cerebral infarction.

Apoptosis in the penumbra is strongly depressed after the onset of angiogenesis. However, the precise mechanism of the role of angiogenesis to prevent apoptosis is still unclear. Furthermore, little is known about the effects of suppression of angiogenesis on the repair process in cerebral infarcted lesions.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method (TUNEL) has been widely used for detecting the DNA fragment of apoptotic cells. Recently, anti-ssDNA antibody has been introduced for a new immunohistochemical evaluation of apoptotic cells, and it has been shown to be as useful as the TUNEL method. The antibody detects the apoptosis of the hippocampal CA1 neuron, therefore, we used this antibody to check the possibility of the apoptosis of neurons in the peripheral area in cerebral infarction.

Cyclophosphamide, as an immunosuppressant and an anti-cancer drug, can inhibit the mitosis of the endothelial cells, resulting in the suppression of angiogenesis. In addition, Cyclophosphamide has been recently clinically used as an anti-angiogenic drug in cancer patients. Therefore, this drug was used to inhibit angiogenesis in the present study.

The present experiment is thus designed to comparatively assess a variety of common cell and tissue reactions in the healing process of thromboembolic rat cerebral infarcts in the presence of suppression of angiogenesis produced by anti-cancer drug administration.

### Materials and Methods

#### Animal

Wistar strain, male 9-10 week old rats (250-300 g) were fed at lib in their cages until use.

#### Experiments

This project was approved by and followed the guidelines published by the Animal Care and Use Committee of Tokyo Medical University.

Two experimental groups were made. One was the angiogenesis suppression group, which was made by intraperitoneal administration (50 mg/kg) of cyclophosphamide (CP) (Shionogi, Tokyo) two days before the operation, defined as the CP-treated group in this study. The other group was not given any drug and was defined as the CP-untreated group. The control normal sham rat group received the same amount of normal saline injections.

Cerebral infarction was accomplished according to the homologous thromboembolic cerebral infarction model. Briefly, animals were anesthetized with ether, and 0.1 ml of blood was obtained by cardiac puncture with a 27 gauge needle and stored for clot formation at room temperature for 48 hours. At the time of operation, the animals were again anesthetized with Nembutal (2 ml/kg). The right common carotid artery was surgically exposed and about 0.1 ml emboli suspended in saline were injected into the carotid artery.

CP-treated rats were killed chronologically by transcardiac perfusion with 15% formalin under ether anesthesia, at 8 hours (number of animals: n = 3), 1 day (n = 3), 2 days (n = 4), 3 days (n = 4) and 5 days (n = 5). In addition, CP untreated rats were killed in the same way at 8 hours (n = 5), 1 day (n = 4), 2 days (n = 5), 3 days (n = 5) and 5 days (n = 5).

#### Tissue preparation

The brains were removed, cut coronally and embedded in paraffin, then 6 μm-thick serial sections were made. Each one of the sections was stained by hematoxylin-eosin (HE).

#### Immunohistochemistry

The avidin-biotin peroxidase complex method (ABC
method) was used for immunohistochemical procedures. Antibodies used were TGF-beta (sc-146, polyclonal, Santa Cruz Biotechnology, CA, USA), anti-VEGF (c-1) (sc-7269, monoclonal, Santa Cruz Biotechnology), Anti-single stranded DNA (A4506, polyclonal, Dako, Kyoto, Japan) and anti-GFAP (M761, monoclonal, Dako).

Briefly, each deparaffinized and rehydrated section was incubated in 0.3% H$_2$O$_2$ in 200 ml methanol in order to inhibit endogenous peroxidase activity for 15 minutes, then heated in 0.01 M citrate buffer solution in a microwave for 30 minutes, then cooled to room temperature (this step was unnecessary for ssDNA stain). After washing in 0.01 M phosphate-buffer saline (PBS), sections were treated for either anti-VEGF (c-1) (1:100), anti-GFAP (1:200) and anti-single stranded DNA (1:100) and incubated overnight at 4°C. After washing in PBS, each section was incubated for 15 minutes with biotinylated linked anti-mouse and anti-rabbit Ig for LSAB 2 system (Dako). Then, each section was washed in PBS again, and incubated for 30 minutes in streptavidin-HRP for LSAB 2 system HRP (Dako). Then each section was immersed for 5 minutes in DAB (3,3-diaminobenzidine tetrahydrochloride-Tris, Muto, Tokyo, Japan) with 0.3% H$_2$O$_2$ drops in 50 ml distilled water. After washing in distilled water, they were counterstained with methyl green for 2 days and observed with a light microscope.

Double immunostaining was also carried out as follows: After the first anti-VEGF or anti-GFAP immunohistochemical process, the sections used for the second immunoreactive process were washed three times for 30 minutes each with 0.1 M glycine-HCL buffer (pH 2.2) to remove the antibody. After rinsing with PBS, the sections were incubated with anti-GFAP or anti-VEGF bodies reversed at 4°C temperature overnight. They were washed with TBS (Tris buffered saline, Dako) again and incubated with ALP (alkaline phosphatase conjugated streptavidin, Biogenex, San Ramon, CA, USA) for 30 minutes, and chromogen by New Fuchsin (Dako).

Histochemical stainings

Although the number of foamy macrophages was counted on HE slides, Sudan III staining method was used for the evaluation of the degree of phagocytosis of foamy macrophages. Sudan III stained sections were made as follows. Fixed tissues were incubated in 0.88 M gum sucrose solid overnight. Then the frozen sections were made. After drying, the sections were washed by 50% alcohol, then they were incubated in Daddi solid for an hour at room temperature. Then sections were washed by 50% alcohol again and counterstained with hematoxylin.

In addition, the Watanabe silver impregnation method was used for the detection of newly formed blood vessels in the lesions.

Statistical analysis of number and luminal volume of newly formed blood vessels

Five representative fields per 0.1 mm$^2$ were chosen from the peripheral area in each section. Regardless of the size and shapes (for example, round, oblong, curved, ovoid, short, long or straight), all newly formed blood vessels within the total five fields were evaluated. The number of microvessels was counted in the fields and then the average density per one field was shown as the number of microvessels/mm$^2$.

In addition, the luminal border of all microvessels in the fields was automatically outlined and the percentage of total vascular lumen area per one field was counted using the IPAP-win system (Sumika Technoservice, Tokyo, Japan).

Statistical analysis of number of TGF-beta 1, VEGF, ssDNA-immunoreactive cells

Total numbers of TGF-beta 1, VEGF, ssDNA immunoreactive cells were counted in the same way and in the same fields mentioned above.

Statistical evaluation

All values are presented as means±SE. The numbers of cells were compared at different time points using Student’s t-test. A value of $p<0.01$ was considered to indicate a statistically significant difference.

Western blot analysis

Hemi-side injured or normal cerebellum was homogenized by a polytron on ice in the lysis buffer (1% sodium dodecyl sulfate, 50 mM Tris-HCl [pH7.6], 150 mM NaCl, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA) and 25 μg/mL leupeptin). The homogenates were first centrifuged at 14,000×g, and supernatants were transferred to fresh tubes for further centrifugation (14,000×g). The obtained clear lysates were dissolved in sample buffer and loaded on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) after the protein assay, and blotted onto the polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) using a semi-dry electrophoretic transfer system (Atto, Osaka, Japan). Then, the first antibody (anti-TGFbeta 1 and anti-VEGF) was loaded onto the membrane after blocking by BlockAce® (Dainippon, Osaka, Japan) at 4°C for overnight and then horse radish peroxidase-conjugated signals were enhanced by an ABC kit. The bands were detected by enhanced chemiluminescence (Immunostar Reagents, Wako) and visualized by a light-capture system (Atto).

Results

Macrophages

The average number of macrophages was counted in
Fig. 1 Appearance of macrophages in the peripheral areas. A–D: CP-untreated group, E–H: CP-treated group. (A, E: 2 days, HE) Macrophages were not evident, although necrotic cells with condensed and fragmented nuclei were present in both groups. (B, F: 3 days, HE) Foamy macrophages were observed along with newly formed microvessels. Both macrophages and microvessels were more numerous in the CP-untreated group. (C, G: 5 days, HE) Full blown foamy macrophages and “mother” vessels with enlarged lumens were observed in the CP-untreated group, whereas macrophages were still not fully foamy with relatively small amounts of cytoplasm in the CP-treated group. Blood vessels were small and narrow in the CP-treated group. (D, H: 5 days, Sudan III) Macrophages in the CP-untreated group contained abundant lipid droplets in the foamy cytoplasm, while macrophages in the CP-treated group contained much less amounts of lipids. Bar=50 μm.
one area of 0.1 mm² in the peripheral areas on HE stained slides.

The lesions became necrotic, showing changes such as eosinophilic cytoplasm, nuclear condensation and fragmented bodies at 2 days in the central core areas in both groups (Fig. 1 A, E).

On day 3, unequivocal macrophages were detected, predominantly around newly formed thin-walled microvessels in the CP-untreated group (275.54 ± 32.32 cells/mm², n = 25). On the same day, macrophages in the CP-treated group were significantly fewer (93.1 ± 36.61 cells/mm², n = 20), (p < 0.01).

On day 5, there was a marked increased number of macrophages in the CP-untreated group (768 ± 64.5 cells/mm², n = 25). At that time, they became fully foamy with abundant lipids in their cytoplasms. In contrast, the macrophage proliferation in the CP-treated group was weak and the number was much less, (294.4 ± 31.01 cells/mm², n = 25) (p < 0.01). Furthermore, many macrophages appeared to be not fully developed (Fig. 1G). Sudan III stain confirmed the observation that macrophages in CP-untreated group were more numerous and had more evident foamcytoplasm, filled with abundant lipid granules (Fig. 1D, H). In addition, there were no macrophages in the sham control group. These data are shown in Fig. 2.

Angiogenesis

The number of microvessels was found to be decreased at 8 hours, compared with the sham control 144.0 ± 37.52 folds/mm² (n = 15). At 2 days, no active angiogenesis was observed and the numbers of microvessels remained low in both groups (Fig. 3 A, E). Unequivocal angiogenesis was observed for the first time at 3 days with the appearance of many microvessels with small and narrow lumens in the CP-untreated group (194.6 ± 53.6 folds/mm², n = 25) (Fig. 3 B). However, the angiogenesis was so weak that the number of blood vessels remained low (99.2 ± 34.68 folds/mm², n = 20) at 3 days in the CP-treated group. (p < 0.01) (Fig. 4 A).

On day 5, angiogenesis became evident in CP-treated group too. However, the degree and pattern of vascular proliferation were quite different between the two groups. In the CP-untreated group the blood vessels tended to have very large lumens with thin walls (Fig. 3C), whereas in the CP-treated group the lumens of vessels still remained small and were not expanded.

The percentage of the lumens area gradually increased from 2 days in both groups, although there was a conspicuous difference at 5 days between the CP-untreated group (30.4 ± 4.21%, n = 25) and the CP-treated group (11.4 ± 3.2%, n = 25) (p < 0.01) (Fig. 4B). The blood vessels tended to have enlarged lumens in the CP-untreated group, resulting thus in the relatively high number of lumens, compared with the CP-treated group, although the number of blood vessels was about the same as the CP-treated group at 5 days. The develop-
Fig. 3  Degree and appearance of newly formed blood vessels and in the peripheral area.  A-D : CP-untreated group, E-H : CP-treated group.  (A, E : 2 days, Silver stain) Proliferation of blood vessels was not evident in both groups.  (B, F : 3 days, Silver stain) Many small blood vessels with narrow lumens were observed in the CP-untreated group, whereas the vascular proliferation was obviously suppressed in the CP-treated group.  (C, G : 5 days, Silver stain) The lumens of blood vessels were very large in the CP-untreated group, whereas the vascular lumens remained small and narrow in the CP-treated group.  (D, H : 3 days, ssDNA immunostain) Apoptotic cells remained relatively few in the CP-untreated group, whereas they were still abundant in the CP-treated group.  Bar=100 μm.
Fig. 4  ■ Express the CP-treated group, □ express the CP-untreated group, □ express the sham control group. (A) Average number of microvessels per 0.1 mm² in the peripheral area. A significant increase was observed between 2 days and 3 days in the CP-untreated group, compared with the CP-treated group; *, p < 0.01. Otherwise, there was no significant difference between the two groups throughout the experiment. (B) Average percentage of vascular lumens in the peripheral area. There was a progressive expansion of vascular lumens in both groups, but the CP-untreated group showed a rapid significant expansion at 3 days and thereafter, compared with the CP-treated group in the same time period; *, p < 0.01.

ment of angiogenesis in the CP-treated group was apparently delayed, compared with that of the CP-untreated group.

**Immunohistochemistry of TGF-beta 1**

TGF-beta 1 immunoreactive cells could be detected in the early stage not only in the peripheral areas but also the core areas, although the immunoreactive cells appeared to be mainly microglia/macrophages around the peripheral areas.

The maximum number of positive cells was obtained at 2 days in the CP-untreated group (230.4 ± 26.77 cells/mm², n = 25), and at 3 days in the CP-treated group (182 ± 53.94 cells/mm², n = 20). In addition, the immunoreactive cells were also detected, albeit only a few, in the sham control group (58.6 ± 28.01 cells/mm², n = 15).

The present results showed that CP administration had no significant influence on TGF-beta1 (Fig. 5).

**Double immunohistochemistry of VEGF / GFAP**

VEGF positive cells were shown to be GFAP negative, thus indicating that VEGF positive cells could be considered to be mainly microglia, endothelial cells and macrophages. Microglia showing the strongest reactivity were mostly located in the areas between the outer astrocyte layer and inner macrophage layer of the lesions.

The immunoreactive microglia appeared from 2 days both in the peripheral area in both groups. The VEGF immunoreactive microglia were more numerous in the CP-untreated group and progressively increased thereafter (Fig. 6).

VEGF immunoreactive cells were not observed in core areas of both groups or in the sham control group.

**Immunohistochemistry of ssDNA**

No ssDNA immunoreactive cells were detected everywhere in both group at 8 hours. At day 1, the im-
Fig. 5 Number of TGF-beta 1 immunoreactive cells. ■ Express the CP-treated group, □ express the CP-untreated group, □ express the sham control group. The maximum number was observed at 2 days in the CP-untreated group and at 3 days in the CP-treated group, respectively.

Fig. 6 Number of VEGF immunoreactive cells in the peripheral area. ■ Express the CP-treated group, □ express the CP-untreated group. They appeared for the first time at 2 days and they tended to be more numerous in the CP-untreated group at all times. However, there was no statistically significant difference.

Fig. 7 Numbers of ssDNA immunoreactive cells. ■ Express the CP-treated group, □ express the CP-untreated group. They were maximum at 1 day in both group and since then they rapidly decreased in both groups, too. However, the degree of the decrease was more remarkable in the CP-untreated group from 2 days to 3 days, compared with the CP-treated group; *, p < 0.01.

Immunoreactive cells were observed in the peripheral area. Nuclei showed an advanced degree of morphological alteration with shrinkage at day 2 in both groups. On day 3, the immunoreactive cells were few in the CP-untreated group (27.6±37.32 cells/mm², n=25) (Fig. 3 D). On the other hand, numerous immunoreactive cells with pyknotic nuclei were seen in the CP-treated group (99.2±20.86 cells/mm², n=20) (p<0.01) (Fig. 3 H). They were very few in both groups at 5 days. The data were shown in Fig. 7.
Fig. 8 Expression of TGF-beta 1 and VEGF detected by Western blot analysis. Actin was also detected. The bands were visualized by a light-capture system, and the expression level at each time point was analyzed. PO represented the band as a positive control. Data represent two independent experiments.

**Western blot analysis of TGF-beta 1**

For further analysis of TGF-beta 1 and VEGF, the total infarct brain was subjected to Western blot analysis. As shown in Fig 8, no clear change of expression of VEGF was observed on non treated group of CP until 24 hours at least. This result is consistent with the result of immunohistochemical analysis. However, the remarkable change of expression of TGF-beta 1 was observed from 8 hours. These lines of evidence indicate that TGF-beta 1 plays a crucial role in the early stage of chronic infarction, but not VEGF.

**Discussion**

The present study showed that an increased number of capillaries were closely related with increased immunoreactivity of TGF-beta 1 and VEGF in CP-untreated rats. Furthermore, blood vessels tended to have enlarged lumens in CP-untreated rats. On the other hand, the lumens of newly formed blood vessels were small and narrow in CP-treated rats, suggesting that CP had an anti-proliferation effect on the vascular cells. In addition, VEGF immunoreactivity was considerably decreased in the blood vessels of CP-treated rats. These findings clearly indicate that these angiogenic chemical factors cooperatively mediate the proliferation of blood vessels.

It is shown that the total number of peripheral blood cells was decreased after CP administration27. Moreover, leukopenia was observed in experimental rat cerebral infarction and should be ascribed to bone marrow suppression, which is apparently induced by administration of anti-cancer drugs28. On the other hand, since macrophages can produce a wide variety of angiogenic factors29,30, it is possible to consider that the decreased number of macrophages could also result in the suppression of angiogenesis. In addition, there is also evidence in the present study that CP could have suppressed the function of TGF-beta 1. If so, since TGF-beta 1 can stimulate the infiltration of macrophages31, it is reasonable to consider that the decreased numbers of macrophages in CP-treated rats could be partly or indirectly ascribed to the suppression of TGF-beta 1 functions.

It seems reasonable that the decreased number of macrophages could directly causes a prolonged delay in the removal of apoptotic cells as well as necrotic tissue. Thus, we can consider that the presence of not fully developed macrophages in CP-treated rats is clearly related to the persistent residual necrotic debris in the center as well as apoptotic cells in the periphery, along with the suppressed angiogenesis.

Macrophages may have multiple sources of origin, including blood borne monocytes, pericytes and intrinsic microglia in cerebral infarcts32-35. Regardless of the source of origin, however, mature macrophages display very similar morphologic features and their source cannot be differentiated by light microscopy. In this study, we attempted to identify foamy macrophages on the basis of morphologic appearance only, and this approach seems acceptable in the present study because our purpose with regard to macrophages was to evaluate whether or not there is any correlation of vascular proliferation and the number and development of foamy macrophages in the lesions.

It is widely anticipated that modulation of angiogenesis will provide therapeutic benefit. For example, there is evidence that the introduction of new blood vessels will benefit patients with circulatory disorders2,5. Encouraging results have been reported in cerebral infarction animals treated with angiogenic factors, which showed an improvement of microcirculation and reduction of infarcted volume36. On the other hand, a wider therapeutic window could be obtained by blocking the apoptotic cascade by using neuroprotective interventions. In fact, CP has an immunosuppressant effect similar to FK506, which has was a potential to preserve neurons from apoptotic death after injury27. In the
The present study, however, we could not prove that CP has a potential to preserve neurons from apoptotic death in the penumbra.

The effects of anti-cancer drugs in ischemic brain damages have not been extensively investigated clinically or experimentally until now. However, one can easily predict that the incidence of ischemic brain injuries could be increased among cancer patients as cancer patients can live longer through the introduction of more effective cancer therapies. Anti-cancer drugs such as CP are known to induce certain degrees of suppression on angiogenesis^38,39^). In treating such patients, therefore, it is necessary to pay attention to the existence of suppressed angiogenesis and its related events in the ischemic brain lesions. Under such circumstances, one may attempt to improve the blood circulation in the lesions, mainly because an enhanced angiogenesis is believed now to hold therapeutic potential for cerebral infarction. However, based on the present results, we would like to consider that the persistent necrotic/ degenerated tissues due to suppressed macrophagic response could potentially be recycled for tissue repair, which allows us to have a chance to widen and lengthen therapeutic window. Further studies are certainly required to prove whether or not a trial of the enhancement of angiogenesis is definitely effective in the treatment of ischemic brain injuries.

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実験的血栓塞栓性脳梗塞核における血管新生抑制の意義

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【要旨】実験的脳梗塞後 2-3 日目の病巣周辺を中心に血管新生が見られたが、この治癒の現れである血管新生の役割について不明な点が多い。そこで、我々は梗塞核における血管新生を抑制することでその新生血管の役割について検討した。9〜10週齢の雄 Wistar ラット（200-250 g）を用い、血栓塞栓性脳梗塞ラットモデルを利用し、梗塞核を作製した。血管新生阻害剤として抗腫瘍 Cyclophosphamide (CP) を使用した。CP 投与群と CP 非投与群の動物を 3〜4 匠ずつ梗塞後 8 時間、1 日、2 日、3 日、5 日目に麻酔下で脳のホルマリン灌流固定法にて殺した。抗 TGF-beta 1 抗体、抗 VEGF 抗体、抗 ssDNA 抗体を用い、ABC 法にて免疫組織化学的染色を行った。各染色標本において梗塞核周辺部と正常対照部から各 5 個所選び、それぞれの陽性反応について、値土標準偏差（SE）で示し、Student’s t 検定法を用い、危険率 1% 以下を有意差ありと判定した。また、TGF-beta 1 と VEGF については Western blot analysis を行った。血管新生は両群共に 2〜3 日目から目立ち、3 日目著しい増殖を示したが、血管数は CP 非投与群に有意に多かった。そして、CP 投与群における新生血管は単層細胞の壁と内腔の微細な小血管が目立った。ssDNA 陽性のアポトーシス細胞は、CP 投与群で有意に多く残存していた。病巣内 Macrophage 数は CP 投与群で全経過を通じて少なく、その大半は不成熟な形態であった。この CP 投与群における macrophage 数の減少は、CP の骨髓機能や血管新生への抑制作用によると考えられる。つまり、macrophages の起源細胞である単球や血管周辺細胞の減少に起因すると考えられる。そして、その数的減少と貪食作用の減弱がアポトーシス細胞の清掃抑制に関与していると考える。超急性期以後の脳梗塞の治療法として、血管新生の抑制を導くと同時に、残存する細胞・組織の再生・再利用を促す方法も考慮すべきと考える。

Key words 血管新生、マクロファージ、アポトーシス、実験的脳梗塞、ラット、シクロホスファミド