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1．研究テーマ

抗血栓薬における脳出血のメカニズム解明と脳出血抑制法の探索

2．本年度の研究業績

(1) 学会・研究会等における発表 (有)・無 (学会名・演題)

(1) 学会名： 第 74 回日本薬理学会総会.

演題： ヘパリンによる脳出血をラジカルスカベンジャーが抑制するーウサギ中大脳動脈血栓モデルを用いてー.

(2) 学会名： XXth International Symposium on Cerebral Blood Flow, Metabolism and Function & Vth International Conference on Quantification of Brain Function with PET.

演題： The Combination of EPC-K1 and Heparin Enhances Neuroprotection in the Rabbit MCA Occlusion Model.

(3) 学会名： 第 75 回日本薬理学会総会.

題名： ヘパリンによる脳出血における内因性 tPA および matrix metalloproteinases の役割：tPA ノックアウトマウス中大脳動脈閉塞モデルでの検討.

(2) 学会誌等に発表した論文 (有)・無 (雑誌名・論文名)

(1) Combination of a Free Radical Scavenger and Heparin Reduces Cerebral Hemorrhage After Heparin Treatment in a Rabbit Middle Cerebral Artery Occlusion Model. Stroke 32 (9): 2157-2163, 2001.

(2) Cerebral hemorrhage due to heparin limits its neuroprotective effects: studies in a rabbit model of photothrombotic middle cerebral artery occlusion. Brain Res. 902 (1): 30-39, 2001.

(3) A novel MCA occlusion model of photothrombotic ischemia with cyclic flow reductions: development of cerebral hemorrhage induced by heparin. Brain Res Prot. In press.

(4) Tissue-type plasminogen activator has paradoxical roles in focal cerebral ischemic injury by thrombotic middle cerebral artery occlusion with mild or severe photochemical damage in mice. J Cereb Blood Flow Metab. In Press.

3．今後の研究計画

脳梗塞の治療薬である抗血栓薬の大きな問題は副作用としての脳出血である。しかし、脳出血のメカニズムはほとんど解明されておらず、臨床での使用の際には脳出血を予知することは不可能である。そこで、我々は抗血栓薬の脳梗塞縮小効果と副作用である脳出血のリスク評価を一緒にできるモデルを確立した。

さらに、我々はt-PA (tissue-type plasminogen activator) とMMP (matrix metalloproteinases) という蛋白は脳出血に大きく関与していることを見つけて、それらの発現を分子薬理学的に経時的に追っていくことで局所の病態を詳細に検討する。

いまは、ヘパリンによる脳出血における t-PA と MMP-9 および MMP-2 の関連を in situ hybridization による mRNA レベルで検討している。さらに、抗酸化剤と抗血栓薬との併用で脳出血が抑制できることを我々は見つけたので、抗酸化剤を用いて t-PA と MMP の活性がどのように修飾されるか検討する。

4．指導責任者の意見

趙氏は当薬理学、大学院博士課程３年生です。彼のテーマは、脳梗塞治療薬における脳出血のメカニズム解明とその予防方策の探索です。１３年度には、日中医学協会から助成を頂きこのテーマで最も重要となる動物モデルの確立を成し遂げました。学会にも演者として積極的に発表し、有名英文雑誌にファーストオーサーとして掲載されました。現在、更に詳細なメカニズムを分子生物学的手法を用いて検討中です。大学院生としての研究期間は後１年ですが、さらなる発展が期待できる大変優秀な研究者と評価しております。

指導責任者氏名 [signature] 印

5．研 究 報 告 書

別紙報告書作成要領により、添付の用紙で研究報告書を作成して下さい。

研究発表中または研究中の本人のスナップ写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

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-日中医学協会助成事業-

-血栓性脳梗塞モデルにおいてヘパリン投与により引き起こされる脳出血の検討-

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ABSTRACT

Intracerebral hemorrhage is the major complication associated with antithrombotic and thrombolytic therapy. Despite efforts directed toward achieving hemorrhagic infarction, an ideal animal model of cerebral hemorrhage has not yet to be established. Using the photothrombotic technique in rabbits, we developed a model of cerebral hemorrhage by inducing cyclic flow reductions in the middle cerebral artery (MCA). Furthermore, the hemorrhage increased 4-fold after infusion of heparin at a dose prolonging activated partial thromboplastin time by about 3 times that of control animals. The photothrombotic occlusion of the MCA is based on a thrombosis induced by endothelial injury through singlet oxygen produced by Rose Bengal injection and green light irradiation. Using a pulse Doppler flowmeter, spontaneous reperfusion of the MCA after the thrombotic occlusion following cyclic flow reductions was observed within 2 h in the majority of animals. This model is unusual with respect to the development of clinical stroke, because of the MCA cyclic flow reductions. Thus it is different from permanent or ischemia/reperfusion MCA occlusion in rodents and may be suitable for studying hemorrhagic risks associated with the use of antithrombotic agents.

Key Words　Cerebral hemorrhage, heparin, photothrombotic occlusion, antithrombotic agents, cerebral ischemia, cyclic flow reductions

INTRODUCTION

Intracerebral hemorrhagic transformation are feared events that may follow antithrombotic and thrombolytic therapy in acute stroke, so high dose use of these agents has been limited [1].

To investigate the efficacy and safety of antithrombotic agents for treatment of stroke, ideal animal models of cerebral infarction with a good reproducibility, which are similar to the clinical situation, are required. Up to date, a variety of methods have been developed for this purpose by many investigators, but they are not yet satisfactory [2].

Based on photothrombotic technique, we developed a new model of intracerebral hemorrhage induced by antithrombotic agents after the rabbit MCA thrombotic occlusion [3].

Heparin is routinely used to prevent recurrent embolism and deep venous thrombosis [4]. Therefore, in the present study, we investigated the efficacy of heparin on cerebral ischemic damage in a rabbit model of MCA photothrombosis and in the same model, cerebral hemorrhage induced by heparin as its side effect was also investigated.

MATERIALS AND METHODS

1. Animals

Male Japanese white rabbits weighting 2.0-3.0 kg. Animals were housed individually in a cage with access to food and water on a 12 h light-dark cycle for one week.

2. Animal preparation

The experimental protocol was approved by the Hamamatsu University School of Medicine Committee on Ethics of Animal Experimentation, and extra care was taken to avoid animal suffering.

Male Japanese white rabbits weighting 2.0-3.0 kg were used. After one week of acclimation, the animals were anesthetized with 1-2% isoflurane (Dainnihon Pharmaceutical, Tokyo, Japan) in 30% O_2 and 70% N_2O using a face mask. Body temperature was maintained at 38℃ with a heating pad (K-module model K-20, American Pharmaseal Company). The right femoral artery was cannulated for blood sampling and the continuous recording of artery blood pressure and heart rate. Artery blood gases were monitored using a gas analyzer (model 860, Ciba-corning, MA, USA) just before and 2 h after the occlusion of the MCA.

A curved skin incision along the rim of the left orbital bone was made, the temporalis muscle was removed with a bipolar electric coagulator (model 80-1160, Valley Forge Scientific Corp., USA) and thermoknife. Under an operating microscope (model KOM 300, Konan Inc., Japan), a oval bony window was opened using a dental drill (model PAL-7, Morita, Japan); the dura mater was intact. With the help of the operating microscope, the MCA and the olfactory tract were identified. The irradiation with green light was directed by a 3-mm-diameter optic fiber mounted on a micromanipulator. The head of the optic fiber was placed on the dura matter just above the MCA where it passes over the olfactory tract , providing an irradiation dose of 0.170 W/cm^2 (wavelength, 540 nm). Intravenous injection of Rose Bengal (10 mg/kg body weight for 3 min through a peripheral ear vein) and photo-irradiation were started simultaneously. The photo-irradiation was continued for 60 min. The local blood flow in MCA was monitored continuously for 2 h after the injection of Rose Bengal using a pulse Doppler flowmeter (PVD-20, Crystal Biotech). The time taken from the start of photo-irradiation to the cessation of blood was regarded as the MCA occlusion time. After the closing of surgical wounds, animals were allowed to recover from the anesthesia.

After the scoring of clinical outcome at 24 h after photothrombosis, animals were anesthetized with an overdose of pentobarbital sodium and then immediately perfused transcardially with normal saline. Twelve consecutive coronal sections were cut from each cerebrum using a slicing apparatus (RBM-7000C, Activatinal Systems INC., Michigan, USA). All coronal sections were photographed immediately. Thereafter, the brain slices were stained with 1% triphenyltetrazolium chloride (TTC) for 30 min, fixed with buffered formaldehyde (pH 7.2) for 24 h and photographed again. The cerebral hemorrhage size and the infarct volume were measured using a computerized image analysis system (NIH Image 1.62 Program, Internet).

3. Administration of heparin and analysis of activated partial thromboplastin time

Heparin was diluted with saline and infused by infusion pump (model KDS-230, USA) at a delivery rate of 0.5 ml/kg/hr. In heparin-treated animals (n = 7), heparin was administered intravenously by injection as a bolus of 100 IU/kg followed by continuous infusion at 75 IU/kg/hr to 24 h, starting 2 h after the start of photo-irradiation. The vehicle-treated animals (n = 14) were infused continuously with saline.

Activated partial thromboplastin time (aPTT) was determined using an automatic Coagulometer KC 4 A (Heinrich Amelung, Germany) as previously described [3].

4. Examination of neurological deficits

At 24 h after photothrombosis, the neurological deficits of each animal were evaluated in a blind manner:

- Wryneck test: the animal was placed in a cage and the torsion of the neck was observed. Behavior was scored as follows: 0, normal; 1, twist of the neck.
- Righting reflex test: the animal was placed on its back, and scored as follows: 0, righted within 1 s; 1, righted within 5 s; 2, did not right within 5 s.
- Dysfunction of paws: the fore paw or hind paw was pulled toward the body. The time to re-extend the paw was scored as follows: 0, achieved within 1 s; 1, achieved within 5 s; 2, not achieved within 5 s.
- Postural reflex test: the animal was pushed in the contralateral direction and scored as follows: 0, normal; 1, reduced resistance to lateral push; 2, fell down on the contralateral side.

5. Data analysis

Data are represented as the mean ± S.E.M. Parameters of cerebral blood flow and cerebral hemorrhage were analyzed by the two-tailed unpaired Student's t-test. The correlation between infarct volume and neurological score was analyzed using linear regressions. A P value < 0.05 was considered significant.

RESULTS

1. Experimental Conditions

All physiological parameters were within the normal range after the photothrombotic occlusion of the MCA.

In heparin-treated animals, aPTT was prolonged markedly at 24 h and about 3 times that of the vehicle group.

The green light irradiation system used in this study is a short-arc type xenon lamp (wavelength 540 nm, bandwidth 80 nm) which has a heat-absorbing filter and a green filter. The xenon lamp irradiation system did not heat the irradiated tissue near the MCA during photo-irradiation.

2. Blood flow in the MCA

The blood flow in the MCA was reduced to zero by the formation of a thrombus at 9.23 ± 0.58 min after Rose Bengal injection. Spontaneous reperfusion of the occluded MCA was observed within 2 h in 95.2% of animals. Spontaneous reperfusion of the MCA after the thrombotic occlusion following cyclic flow reductions was observed.

3. Cerebral hemorrhage volume as well neurological deficit

Infarct volume was 219.0 ± 14.1 mm^3 in vehicle-treated animals and 226.6 ± 19.2 mm^3 in heparin-treated animals. Hemorrhage was mainly observed in the basal ganglia in almost of animals. Heparin-treated animals had a 4-fold increase in cerebral hemorrhage compared with the vehicle-treated controls ($p < 0.0001$). Gross hemorrhage in the ventriculus lateralis and the basal ganglia was observed in 3 of 7 heparin-treated animals, while no gross hemorrhage was observed in the vehicle-treated animals.

In the control group, wryneck and contralateral forelimb flexion were frequently observed. A correlation between infarct volume and neurological score was observed ($r = 0.539$, $n = 21$, $p < 0.01$,).

Heparin treatment tended to worsen neurological deficits at 24 h compared with the control group.

DISCUSSION

We have described a simple and reproducible model of heparin-induced cerebral hemorrhage achieved by spontaneous reperfusion of the occluded MCA following cyclic flow reductions. This photothrombotic occlusion model results in a platelet- and fibrin-rich thrombus in the MCA at the irradiated site [3,5]. In this study, the photochemical approach to inducing thrombotic occlusion is based on the injection of Rose Bengal and green light from a xenon lamp irradiation system. Rose Bengal is a photosensitive dye, and its photoactivation produces reactive oxygen species, mainly singlet oxygen by a 'photodynamic Type II' energy transfer [9]. Reactive oxygen species cause endothelial injury followed by the adhering and aggregating of platelets to fibrin nets, and the formation of a platelet- and fibrin-rich thrombus at the irradiated site [10].

The advantages of this model include occlusion of the MCA by a nonmechanical approach, and an intact dura mater to maintain normal intracranial pressure. Another important feature is the continued cyclic flow reductions in the MCA. Therefore, the present system represents a new approach to the occlusion of the MCA different from previously described models of permanent occlusion or ischemia/reperfusion [6-8]. In sixty percent of patients, the middle cerebral artery is spontaneously recanalized in the early phase of ischemic stroke without receiving thrombolytic therapy [11], and rethrombosis after thrombolysis is also frequently observed in the cerebral artery [12,13]. This suggests that cyclic flow reductions occur in the acute phase of human stroke. However, in permanent models [6], suture MCA occlusion models [8,14], and embolic MCA occlusion models [15,16], spontaneous reperfusion has not been observed. Therefore, this model is unusual with respect to the development of clinical stroke, because of the cyclic reductions in blood flow in the MCA.

The other advantages of the proposed model include: (1) A light source that is prefiltered and concentrated by an elliptical reflector that has a special coating for efficient absorption of infrared and ultraviolet radiation. (2) In this model, not only platelet but also fibrin is involved in the formation of the thrombus [10]. Therefore, in this model, a tissue plasminogen activator caused reopening of the occluded MCA and reduced cerebral infarction [10]. Recently, we established a thrombotic occlusion model in which spontaneous recanalization with cyclic flow reductions was observed [17]. Moreover in this model, we have demonstrated that a GPIIb/IIIa antagonist reduced cerebral infarction without enhancing cerebral hemorrhage [18], and a delayed administration of heparin initiated 2 h after thrombosis aggravated significantly cerebral hemorrhage [3]. More recently, we demonstrated that the combination of a free radical scavenger (EPC-K1) and heparin reduced heparin-induced cerebral hemorrhage and enhanced neuroprotection from cerebral ischemic damage in this model [19]. It is therefore suggested that the MCA occlusion model of thrombotic ischemia with cyclic flow reductions developed and reported from our laboratory is useful for investigating efficacies and hemorrhagic risks associated with antithrombotic agents for stroke research.

Our previous results showed that the longer the irradiation, the more continuous the cyclic flow reductions in guinea pig [17], and infarct volume was reduced when cyclic flow reductions through the MCA were prevented in rabbit [18]. In contrast, animals that had more cyclic flow reductions had a significantly larger cerebral hemorrhage volume. Therefore, in this study, we sought to establish a model in which cyclic flow reductions continued after reperfusion of the occluded MCA. Based on our preliminary studies, we used a low dose of Rose Bengal and low intensity irradiation, but a longer

irradiation time to produce a gradual vascular injury; under these conditions, cyclic flow reductions continued and cerebral hemorrhage also occurred.

In conclusion, based on photothrombotic occlusion of rabbit MCA, we have developed a new model of cerebral hemorrhage induced by heparin therapy. Spontaneous reperfusion of the MCA after the thrombotic occlusion following cyclic flow reductions is an important characteristic of this system. This model can be extended to determine the hemorrhagic risks associated with the use of antithrombotic agents.

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注: 本研究は Brain Research Protocols に掲載予定である。

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