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(10)ラット一過性局所脳虚血モデルでのＥＰＣ-Ｋ１の効果

ラット一過性局所脳虚血モデルでのEPC-K1の効果

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［要約］

EPC-K1は抗酸化作用を有する物質であり、虚血性細胞障害を軽減させることが期待されている。そこでわれわれはラット局所脳虚血モデルを用いて脳虚血性障害に対するEPC-K1の効果について検討した。中大脳動脈（MCA）60分間閉塞後に形成された脳梗塞は、EPC-K1投与により有意に縮小された。また、EPC-K1投与群ではアポトーシス関連蛋白であるcaspase-3、DNAの酸化的損傷のマーカーである8-OHdG、TUNELの全てにおいて、それらの陽性細胞はvehicle群に比較して減少した。これらのことより、EPC-K1による酸化的DNA障害抑制が神経細胞死の抑制、および梗塞巣の縮小に関与していたものと思われた。

【目的】　EPC-K1は、vitamin CとvitaminEのエステル化合物であり、抗酸化作用を有することが知られている。生理的状況下においては，スーパーオキサイドはSODやグルタチオンペルオキシダーゼ、カタラーゼにより消去されている。しかし、虚血下においてはフリーラジカルが多量発生し、これらの機構では消去しきれなくなる。そのような理由から、虚血再灌流による脳障害において、ラジカルスキャベンジャーによる脳保護作用が検討され、今まで種々の分子においてその有効性が確認されてきた。われわれはラット局所脳虚血モデルを用いて本薬剤の脳梗塞巣に与える影響と、酸化的DNA障害に与える影響についてTTC染色と8-OHdG免疫染色を行うことにより検索した。さらに、caspase-3染色とTUNEL染色を行うことによってアポトーシスの関与についても検索した。

【方法】　Wistar ratの総頚動脈からナイロン栓子を挿入し右中大脳動脈起始部を60分間閉塞した。脳血流再開の直後と3時間後に、EPC-K1 20mg/kg(n=7)、またはEPC-K1 10mg/kg(n=9)を頚静脈から投与した。vehicle(n=9)群においては生食(1ml/kg)を投与した。脳梗塞巣の定量は、再灌流２４時間後に麻酔下ラットを断頭、脳の２mm厚スライスを作製し、TTC染色を行うことによって判定した。また、再灌流２４時間後にラットの脳を摘出し、10μm厚の凍結切片を作成、この切片を用いてTUNELと免疫組織化学染色を行った。TUNEL染色は-80℃で保存しておいた脳凍結切片を、乾燥状態のまま常温に戻した後、10%中性ホルマリンにて10分間固定した。PBSで洗浄後、H2O2処理し、TdT、ビオチン化dNTPと反応

させた。反応後、streptavidin-horseradish peroxidase(HRP)処理を行い、2,3'-diaminobenzidine tetrahydrochloride (DAB)で発色させた。8-OHdG、caspase-3およびactive caspase-3の免疫組織化学染色においては、1次抗体として抗8-OHdG抗体(MOG-020、JICA、Shizuoka、Japan)、抗 caspase-3 抗体 (sc-1217、Sana Cruz Biotechnology Inc、CA)、抗 active caspase-3 抗体 (CFZ-05、Genzyme/Techne、U.S.A) を用いた。染色は、ABC法を用い、DABを発色物質として行った。

【結果】 脳梗塞巣は、Vehicle群 (230.0±61.2mm^3) に対しEPC-K1 20mg/kg投与群 (156.8±60.5mm^3) で有意に縮小していた。EPC-K1 10mg/kg投与群 (216±78 mm^3)では有意な縮小を認めなかった。組織学的検索では、Sham群の脳では、caspase-3、8-OHdG、TUNEL陽性細胞はいずれも認められなかったが、vehicle 群の梗塞脳では神経細胞の細胞質にてcaspase-3が、細胞核にて8-OHdGが著明に染色され、TUNEL陽性細胞が多数出現していた。一方、EPC-K1投与群ではcaspase-3、8-OHdG、TUNELともに陽性細胞がvehicle群に比較して減少した。

【考察】 抗酸化物質である EPC-K1 の投与により梗塞巣の縮小が認められた。これには、8-OHdG 産生の抑制も認められることから、その抗酸化作用が障害軽減に関与している可能性があると考えられた。一方、caspase-3 陽性細胞、TUNEL 陽性細胞も減少したことから、アポトーシスの抑制も関与している可能性があると思われた。

Glial cell line-derived neurotrophic factor (GDNF) の脳保護作用の治療的有効時間域 (therapeutic time window)および治療 mechanism の検討

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[要約]

GDNF は神経細胞保護作用を有する物質であり、虚血性細胞障害を軽減させることが期待されている。そこでわれわれはラット局所脳虚血モデルを用いて脳虚血性障害に対する本薬剤の脳梗塞巣に対する治療の有効時間域について検討した。中大脳動脈 (MCA) 90 分間閉塞後に形成された脳梗塞は、再潅流直後と 1 時間の GDNF の投与により有意に縮小された。また、再潅流直後の GDNF 投与群ではアポトーシス関連蛋白である active caspase-3 と active caspase-9 の二重免疫蛍光染色、TUNEL の全てにおいて、それらの陽性細胞は vehicle 群に比較して減少し、TUNEL と active caspase-3 の陽性細胞は active caspase-9 のより有意な減少されった。これらのことより、GDNF の保護作用の治療的有効時間域は 1 時間で、この作用は cytochrome c と active caspase-9 の経路に対する弱いと思われた。

【目的】 GDNF は神経細胞保護作用を有することが知られている。我々はラット局所脳虚血モデルを用いて本薬剤の脳梗塞巣に対する治療の有効時間域について TTC 染色を行うことにより検索した。さらに、active caspase-3 と caspase-9 の蛍光二重染色と TUNEL 染色を行うことによって治療効果の mechanism についても検索した。【方法】 Wistar rat の総頚動脈からナイロン栓子を挿入し右中大脳動脈起始部を 90 分間閉塞した。脳血流再開の直後と 1 時間と 3 時間後 GDNF (2.5μg/体重 250g) を投与を行った。脳梗塞巣の定量は、再灌流 24 時間後に麻酔下ラットを断頭、脳の 2mm 厚スライスを作製し、TTC 染色を行うことによって判定した。また、再灌流 12 時間後にラットの脳を摘出し、10μm 厚の凍結切片を作成、この切片を用いて TUNEL と蛍光免疫組織化学染色を行った。TUNEL 染色は-80℃で保存しておいた脳凍結切片を、乾燥状態のまま常温に戻した後、10%中性ホルマリンにて 10 分間固定した。PBS で洗浄後、H2O2 処理し、TdT、ビオチン化 dNTP と反応させた。反応後、streptavidin-horseradish peroxidase(HRP) 処理を行い、2,3'-diaminobenzidine tetrahydrochloride (DAB)で発色させた。Active caspase-3 および active caspase-9 の蛍光二重染色においては、1 次抗体として抗 active caspase-3 抗体 (#IMG-144, San Diego, CA) と抗 active caspase-9 抗体 (#9501S, Cell Signaling Tech, MA) を用いた。二次抗体は Goat anti-rabbit IgG-FITC (#5570500, Biomedical Tech Inc, USA) と Goat

anti-mouse IgG-TRITC (＃108H9165, Sigma Chemical Co, USA)を用いた。

【結果】 脳梗塞巣は、Vehicle群（295.7 ± 56.6, n=7）に対しGDNF投与群は直後（177.0 ± 52.8 mm^3, n=6, $p<0.01$）と1時間後（220.2 ± 51.5, n=7; $p<0.05$）で有意に縮小していた。GDNF 3時間後投与群（250 ± 57.7, n=6)では有意な縮小を認めなかった。組織学的検索では、GDNF直後投与群ではactive caspase-3とactive caspase-9、TUNELともに陽性細胞がvehicle群に比較して明らかに減少した。GDNF3時間後投与群は変化がなかった。蛍光二重染色は細胞中にactive caspase-9の蛋白質がactive caspase-3のより有意な減少がなかった。

【考察】 GDNFの脳保護作用の治療的有効時間は、脳血流再開の直後から投与のが最も有効であって、GDNFの保護作用の治療的有効時間域は1時間で、この作用はcytochrome cとactive caspase-9の経路に対する弱いと思われた。

Attenuation of oxidative DNA damage with a novel antioxidant EPC-K1 in rat brain neuronal cells after transient middle cerebral artery occlusion

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Abstract

EPC-K1, L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl-hydrogen phosphate] potassium salt, is a novel antioxidant. In this study, we investigated a reduction of a oxidative neuronal cell damage with EPC-K1 by immunohistochemical analysis for 8-hydroxy-2'-deoxyguanosine (8-OHdG) in rat brain with 60 min transient middle cerebral artery occlusion, in association with terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick end labeling (TUNEL) and staining for total and active caspase-3. Treatment with EPC-K1 (20mg/kg iv) significantly reduced infarct size ($p<0.05$) at 24 h of reperfusion. There were no positive cells for 8-OHdG and TUNEL in sham-operated brain, but numerous cells became positive for 8-OHdG, TUNEL and caspase-3 in the brains with ischemia. The number was markedly reduced in the EPC-K1 treated group. These reductions were particularly evident in the border zone of the infarct area, but the degree of reduction was less in caspase-3 staining than in 8-OHdG and TUNEL stainings. These results indicate EPC-K1 attenuates oxidative neuronal cell damage and prevents the neuronal cell death.

Abbreviations: ANOVA, two way analysis of variance; CBF, cerebral blood flow; DAB, 2,3'-diaminobenzidine tetrahydrochloride; MCAO, middle cerebral artery occlusion; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; SOD, superoxide dismutase; TTC, 2,3,5 triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick end labeling.

INTRADUCTION

L-Ascorbic acid (vitamin C) and α-tocopherol (vitamin E), two endogenous antioxidants, are important members of non-enzymatic antioxidant defense systems. However, as vitamin E is insoluble in water, its water-soluble analogs are synthesized in order to improve its absorption. EPC-K1, L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-l-benzopyran-6-yl-hydrogen phosphate] potassium salt, is a novel phosphate ester of vitamin C and vitamin E, which is soluble in both water and lipid [1]. It was reported that EPC-K1 could act as a scavenger of hydroxyl radicals and an inhibitor of lipid peroxidation [2]. Hydroxyl radical is highly reactive, and oxidizes cellular lipids, protein and nucleic acids, leading to cell death [3, 4,]. Under physiological condition, superoxide and hydrogen peroxide are constantly scavenged by superoxide dismutase (SOD), glutathione peroxidase, and catalase. Under condition of ischemia, however, an overproduction of oxygen free radicals perturbs the antioxidative defense mechanisms, and hydroxyl radicals are generated. Studies have shown that radical scavengers and inhibitors of lipid peroxidation can ameliorate ischemic neuronal damage [5,6,7,8,9,10,11,12,13,]. Indeed, previous study demonstrated that EPC-K1 reduced ischemic brain damage [14]. However, the mechanism of its neuroprotective effect had been unclear.

In the present study, therefore, we examined the effect of EPC-K1 on ischemic/reperfusion brain injury by measuring infarct volume and performing terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick end labeling (TUNEL) study. Furthermore, we also investigated its effect on oxidative DNA damage and apoptosis by immunohischemical analysis for 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is a oxidative DNA damage marker and caspase-3.

MATERIALS AND METHODS

Adult male Wistar rats (body weight, 270 to 300 g) were anesthetized with an intraperitoneal injection of pentobarbital (4 mg/100 g), and a burr hole with a diameter of 1.5 mm was made in the skull using an electric dental drill with care to avoid traumatic brain injury. The location of the burr hole was 3 mm dorsal and 5 mm lateral to the right from the bregma, which was located in the upper part of MCA territory. Thin bone was preserved in the burr hole. The animals were allowed to recover at ambient atmosphere.

On the next day, at about 24 h after the drilling, the rats were anesthetized by inhalation of a nitrous oxide/oxygen/halothane (69%/30%1%) mixture and the right MCA was occluded by insertion of nylon thread through the common carotid artery according to previous report. Body temperature was maintained at 37 ± 0.3°C during surgical procedure. After 60 min of occlusion, the cerebral blood flow (CBF) was restored by withdrawal of the nylon thread. EPC-K-1 was diluted into 0.9% NaCl containing 1N NaOH, to give a ultimate EPC-K1 concentration of 10 mg/1 ml with pH 7.5. Vehicle group was treated the saline. Animals were infused intravenously with EPC-K1 0.25ml /250g, EPC-K1 0.125ml/250g and same amount of saline through the jugular vein just and 3 h after the reperfusion. Blood samples (90 μl) were collected before and just after MCAO from ventral tail artery for measurement of PO_2, PCO_2, and pH (blood gas analyzer model ABL330, Radiometer). Regional CBF of right frontoparietal cortex region was measured before and just after MCAO, as well as before, just after (0), and at 3 h after the reperfusion. Rectal temperature was monitored continuously with a rectal probe inserted to a 4 cm. The animals were allowed to recover at ambient temperature (21 to 24 °C) until sampling. The experimental protocol and procedures were approved by the Animal Committee of the Okayama University Medical School.

To examine a possible effect of EPC-K1 on infarct size after transient MCAO, forebrains were removed at 24 h of reperfusion and divided into sections of 2-mm thickness. The coronal sections were immersed in 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for

30 min, and their sections were fixed in 10 % neutralized formalin. The infarct areas of each section were measured using Scion Image software, version 1.62a.

For TUNEL study and immunohistochemical analysis, the forebrains were quickly removed at 24 h after the reperfusion and frozen in powered dry ice (n=4 each, in vehicle and 20mg/kg EPC-K1 applied groups). Coronal sections were cut on a cryostat at –25 °C and collected on glass slides coated with silan. Sham control sections (n=2) were also obtained. TUNEL study was performed with use of a kit (TACS TdT in situ apoptosis detection kit #80-4625-00, Genzyme), as our previous report [15]. For immunohistochemical analysis of 8-OHdG, the sections were fixed in ice-cold acetone for 5 min and air-dried, followed by a rinse in phosphate-buffered saline (PBS). After blocking with 2% normal horse serum in PBS for 2 h, the slides were incubated with mouse monoclonal antibody against 8-OHdG (MOG-020, JICA, Shizuoka, Japan) at a concentration of 7.0 μg/mg in 2% normal horse serum and 0.3% polyoxyethelene octyphenyl ether (Triton-X) for 8 h at 4 °C. The specificity of this antibody had been established elsewhere [16,17]. The slides were then washed and incubated for 3 h with biotinylated anti-mouse IgG (BA-2000, Vector Laboratories) for 30 min, and then developed using diaminobenzidine (DAB) as a color substrate. The reaction were stopped by washing them in distilled water. A set of sections was stained in a similar way without the first antibody.

For immunohistochemical analysis for total caspase-3 and active caspase-3, the sections were also fixed in ice-cold acetone, air-dried, and then rinsed 3 times in PBS. After blocking with 2% normal serum for 2 h, the slides were incubated for 16 h at 4 °C with first antibody: a goat polyclonal antibody against total caspase-3 (sc-1217, Sana Cruz Biotechnology Inc, CA) at 1 : 100 or a rabbit polyclonal antibody against active caspase-3 at 1 : 100. A set of sections was treated without the first antibody, in order to ascertain specific finding of the first antibodies. The speciaficity of this antisera had been confirmed elsewhere [18]. The sections were then washed and incubated for 1 h with the biotinylated second antibody (1 : 200), followed by incubation with avidin-biotin-peroxidase complex for 30 min. Diaminobenzidine (DAB) was used as a color substrate. In this study, we performed quantitative analysis as our previous report with slight

modification [15]. Briefly, the number of positive cells were counted in three pixels of 1×1 mm^2. First is a region of cortex 2 mm from the midline rendered ischemia by MCAO. This region falls within the border of infarction in vehicle-treated group, but is spared in EPC-K1-treated group. Second is a region of dorsolateral cortex involved in infarcts in both vehicle- and EPC-K1 group. Third is a region of medial striatum involved in infarcts in vehicle-treated group, but variably involved in EPC-K1 group. These staining were categorized into 4 grades to count in the following: (-) no staining, (+) small (1-25), (2+) moderate (26-70), or (3+) a large (more than 71), respectively (Table).

Statistical analyses were performed using Student's t-test for infarct volume, two way analysis of variance (ANOVA) followed by Bonferroni/Dunnett for infarct area, or ANOVA repeated measure for rCBF data, respectively.

RESULTS

There was no significant difference in physiological parameters between the vehicle- and two EPC-K1-treated groups. rCBF was declined to lesser than 40% of basal level immediately after MCAO, and recovered after reperfusion in vehicle or two EPC-K1-treated group, respectively. There was no significant difference in rCBF between two animal groups.

Infarct area of two sections (8 and 10 mm caudal from frontal pole) of EPC-K1-treated (20 mg/kg) group was significantly smaller ($p<0.05$) than vehicle group (Fig. 1A). The infarct volumes of vehicle-treated and EPC-K1-treated (20 mg/kg) group at 24 h after transient MCAO were 230.0 ± 61.2 (mean $\pm$ SD, n=9) and 156.8 ± 60.5 mm^3 (n=7; $p<0.05$ vs. the vehicle-treated group), respectively (Fig. 1B). Infarct area and volume of 10 mg/kg EPC-K1-treated group were also smaller than vehicle-treated group, but there was no statistical significance between two groups (Fig. 1).

TUNEL and 8-OHdG staining were negative in the sham control brain (Fig. 2a, d), but

became positive in the brain at 24 h of reperfusion (Fig. 2b, e). Positive cells were mainly distributed in the ischemic core, but were also present in the border of MCA area. Approximately 70% of cells were positive for TUNEL and 8-OHdG in that area. No positive cells were found in the contralateral area. Treatment with EPC-K1 (20 mg/kg) markedly reduced the number of both TUNEL and 8-OHdG positive cells, especially in the border of the infarct area (Fig. 2c, f). Total and active caspase-3 staining showed numerous positive cells in the ischemic hemisphere of rats at 24 h of reperfusion, especially in the border of the infarct area (Fig. 2h, k). There were no positive cells in the contralateral hemisphere or in the either hemisphere of sham controls (Fig. 2g, j). Treatment with EPC-K1 (20 mg/kg) greatly reduced the number of positive cells in the ischemic hemisphere, particularly in the border of the infarct area as compared to vehicle-treated group (Fig. 2h, k). The degree of reduction was milder in caspase-3 staining than 8-OHdG and TUNEL stainings (Table, Fig. 2).

DISCUSSION

In this study, we found that EPC-K1 (20 mg/kg) reduced infarct volume when administered intravenously. The mechanism of the protective effect of EPC-K1 after transient MCAO seems to be related with several important findings. First, there is no significant difference between the vehicle and EPC-K1-treated group in the rCBF after transient MCAO and reperfusion. Second, the effect was associated with reduction of 8-OHdG-positive cells. Under a condition of severe oxidative stress, the C-8 position of 2′-deoxyguanosine, which is the constituent of DNA, is hydroxylated, and 8-OHdG is produced [19]. Hydroxyl radical, singlet oxygen, and peroxynitrite are proposed to be responsible for this production [20,21]. In our previous study, the extent of oxidative damage was investigated in rat brain after transient MCAO by immunohistochemical analysis for 8-OHdG [22]. In the current study, we showed that intravenous EPC-K1 (20 mg/kg) decreased 8-OHdG immunoreactivity, especially in the border (penumbra) of the ischemic area

that is spared from infarction. This result shows that EPC-K-1 attenuates oxidative DNA damage induced by the transient MCAO.

On the other hand, abundant evidence suggest that apoptosis plays an important role in cell death after stroke. This includes evidence of DNA fragmentation (laddering) in the ischemic hemisphere, as well as evidence that protein synthesis inhibitors can block cell death, both prominent characteristics of apoptosis [23]. In the current study, we used TUNEL and caspase-3 staining as markers of apoptosis. We found that, consistent with its infarct-reducing effects, intravenous EPC-K1 (20 mg/kg) reduced the number of TUNEL positive cells in the ischemic hemisphere, especially in the ischemic "penumbra" that was spared from infarction. Previous studies show that 90-95% of TUNEL positive cells in the ischemic brain are neurons [24]. Caspases, which are strongly related to the process of apoptotic cell death, were induced and mainly located in the ischemic penumbra, suggesting that the expansion of neuronal cell damage in this area may be primarily by an apoptotic mechanism. Caspase-3, 29-kDa pro-apoptosis cysteine protease, exists in both inactive and active forms. The inactive form of the enzyme is present at very low levels, and the active form is barely detectable in normal rat brain. Following focal cerebral ischemia, caspase-3 immunoreactivity increased in the ischemic penumbra of affected hemisphere [25]. In the current study, we found the reduction of the number of positive cells for both active and total caspase-3 in the border of the ischemic area, although the degree of the reduction was less than 8-OHdG and TUNEL. These results indicate that there are direct or indirect cytoprotective effects of EPC-K1 in the ischemic tissue.

In conclusion, the current study demonstrated that the ameliorative effect of EPC-K1 on ischemic brain injury was associated with reduction of 8-OHdG-, TUNEL-, total and active caspase-3-positive cells.

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Figure legends

Fig. 1) Effects of EPC-K1 (10 mg/kg and 20 mg/kg) on infarct area (A), and infarct volume (B) at 24 h after 60 min of transient MCAO. A, Infarct areas of 2 coronal sections (8 and 10 mm caudal) from EPC-K1 (20 mg/kg) treated group (n=7) were significantly smaller than those of the vehicle group (n=9, $*p<0.05$). B, Infarct volume in the brain sections was significantly reduced by EPC-K1 (20 mg/kg) treatment ($*p<0.05$). There are no significant difference between 10 mg/kg EPC-K1 group and vehicle group.

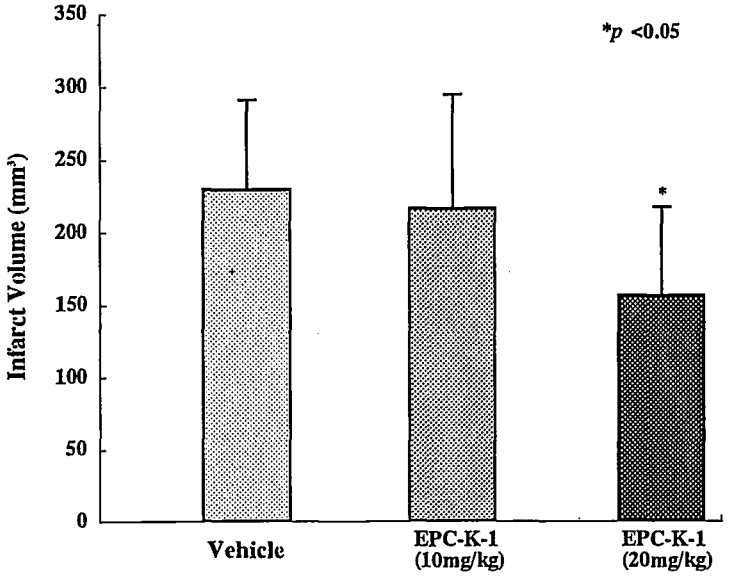
Fig. 2) Representative stainings of TUNEL (a-c), 8-OHdG (d-f), active caspase-3 (g-i) and total caspase-3 (j-l) in sham control brain (a, d, g, j), vehicle (b, e, h, k) or EPC-K1 (20mg/kg) (c, f, i, l) brain, respectively. No cells are stained in the sham control brain (left column). In contrast, large number of staining cells became positive in the vehicle group (middle column), which are reduced in the EPC-K1 (20mg/kg) group (right column). Magnifications, ×50. Bar in panel l, 0.05 mm.

Table. Number of TUNEL and immunohistochemical staining of positive neurons with or without EPC-K-1 (20 mg/kg) treatment after MCA occlusion

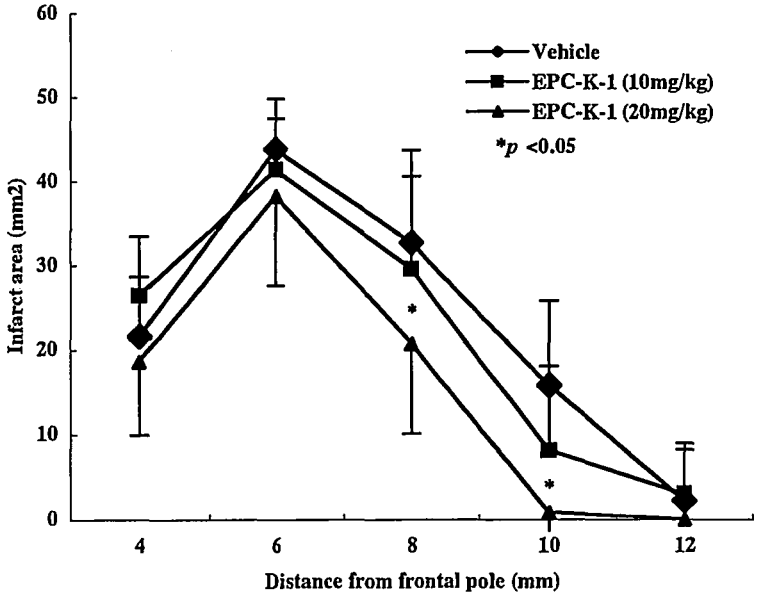
	TUNEL	Active caspase-3	Total caspase-3	8-OH dG
Sham	-	-	-	-
	-	-	-	-
Vehicle				
1	3+	2+	3+	+
2	2+	3+	3+	2+
3	3+	3+	3+	2+
4	3+	3+	2+	2+
EPC-K-1(20 mg/kg)				
1	2+	2+	2+	+
2	+	2+	+	+
3	2+	2+	2+	+
4	+	2+	+	+

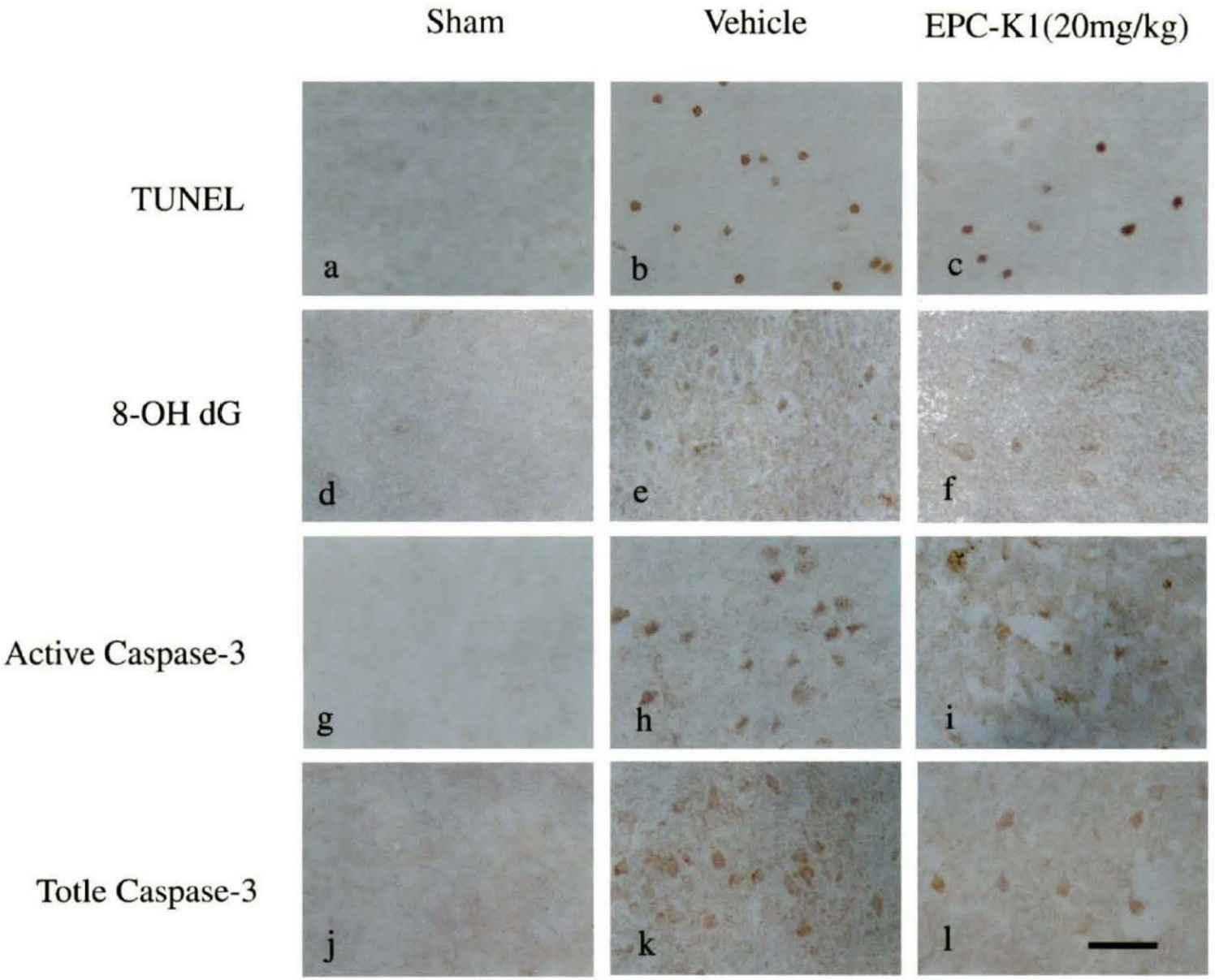
The staining was categorized into the following four grades: no staining, small (1-25), moderate (26-70), or a large (71-100 or more) number of stained cells into (-), (+), (2+), (3+), respectively. Sham control group, n=2, Vehicle and EPC-K-1(20 mg/kg) group, n=4.

A


*p <0.05
Infarct Volume (mm³)
350
300
250
200
150
100
50
0
*
Vehicle
EPC-K-1 (10mg/kg)
EPC-K-1 (20mg/kg)

B


Vehicle
EPC-K-1 (10mg/kg)
EPC-K-1 (20mg/kg)
*p <0.05
Infarct area (mm2)
60
50
40
30
20
10
0
*
*
4
6
8
10
12
Distance from frontal pole (mm)


Sham
Vehicle
EPC-K1(20mg/kg)
TUNEL
8-OH dG
Active Caspase-3
Totle Caspase-3
a
b
c
d
e
f
g
h
i
j
k
l

Time dependent amelioration against ischemic brain damage by glial cell line-derived neurotrophic factor after transient middle cerebral artery occlusion in rat

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Abstract

Time dependent influence of glial cell line-derived neurotrophic factor (GDNF) was examined after 90 min of transient middle cerebral artery occlusion (MCAO) in rats. Treatment with GDNF significantly reduced the infarct volume stained with 2,3,5-triphenyltetrazolium chloride (TTC) when GDNF was topically applied at 0 and 1 h of reperfusion, but became insignificant at 3 h as compared to vehicle group. The protective effect of GDNF was closely related to the significant reduction of the number of TUNEL positive cells as well as immunofluorescently positive cells for active forms of caspases, especially active casapase-3 but not -9.

Thus, the present study showed that topical application of GDNF significantly reduced infarct size in a time-dependant manner, while the therapeutic time window was shorter than other chemical compounds such as an NMDA receptor antagonist (MK-801) and a free radical scavenger (PBN). The effect of GDNF was stronger in suppressing active caspase-3 than active caspase-9.

THEME A: DEVELOPMENT AND REGENERATION

TOPIC: GDNF: Biological effect

Keyword: GDNF, Ischemia, TUNEL, caspase-3, caspase-9

Abbreviations: ANOVA, two way analysis of variance; BDNF, brain-derived neurotrophic factor; CBF, cerebral blood flow; DAB, 2,3'-diaminobenzidine tetrahydrochloride; FITC, fluorescein-isothiocyanate; GDNF, glial cell line-derived neurotrophic factor; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; rCBF, regional cerebral blood flow; TTC, 2,3,5 triphenyltetrazolium chloride; TRITC, tetramethylrhodamine-isothyocyanate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin in situ nick end labeling.

Glial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β superfamily, is a potent neurotrophic factor that promotes the survival and morphological differentiation of dopaminergic neurons [3, 7, 11,16] and motoneurons [13, 18]. GDNF is upregulated after mechanical injury and cerebral ischemia/reperfusion [1, 15]. When cells receive apoptotic stimuli, mitochondria releases cytochrome c which then binds to Apaf-1 together with ATP. The resultant complex recruits caspase-9 leading to its activation. Activated caspase-9 cleaves downstream caspases such as caspase-3, -6 and -7 for initiating the caspase cascade [9], finally induces the DNA fragmentation. Another pathway for caspase-3 activation has also been identified that is independent of mitochondrial cytochrome c release and caspase-9 activation [14].

Others and we have reported that topical application and intracerebral administration of GDNF decreased the size of ischemia-induced brain infarction [8,17]. GDNF decreased ischemic brain edema and the number of TUNEL positive neurons with suppressing apoptotic pathways such as caspases-1 and -3 [2, 8]. However, therapeutic time window is not still clear when GDNF should be administered after ischemia in relation to caspase activations. In this study, therefore, we examined a time dependent influence of GDNF on the infarct size after 90 min of transient middle cerebral artery (MCA) occlusion model in relation to modification of active forms of caspases-3 and -9.

Animal experiment was performed in the similar way to our previous reports [1, 6]. In brief, a burr hole was made in the skull of adult male Wistar rats (body weight, 250 to 280 g) at 3 mm dorsal and 5 mm lateral to the right from the bregma at 24 h before MCA occlusion (MCAO). On the next day, the rats were anesthetized by inhalation of a nitrous oxide/oxygen/halothane (69%: 30%: 1%) mixture and the right MCA was occluded by insertion of a nylon thread with silicon coating through the common carotid artery. Body temperature was maintained at 37 ± 0.3°C during surgical procedure. After 90 min of occlusion, the cerebral blood flow (CBF) was restored by withdrawal of the nylon thread. A

small piece (8 mm^3) of spongel presoaked vehicle (Ringer solution) or GDNF Ringer solution (2.5 μg/9 μl), was placed in contact with the surface of the cerebral cortex, just after, or 1 h and 3 h after the reperfusion. Blood samples (90 μl) were collected just after MCAO and reperfusion from ventral tail artery for measurement of PO_2, PCO_2, and pH (blood gas analyzer model ABL330, Radiometer). Regional CBF (rCBF) of right frontoparietal cortex was measured before, during, and just after MCAO. The animals were allowed to recover at ambient temperature (21 to 24 °C) until sampling. The experimental protocol and procedures were approved by the Animal Committee of the Okayama University Medical School.

At 24 h of reperfusion, the animal forebrains (n=7, 6, 7 and 6, in vehicle, just, 1 h and 3 h after reperfusion of GDNF treatment groups, respectively) were divided into coronal sections of 2-mm thickness, and were stained with 2,3,5-triphenyltetrazolium chloride (TTC), and infarct size was measured [19].

For TUNEL and immunofluorescent analyses, the forebrains were frozen at 12 h after the reperfusion (n=3, both vehicle and GDNF applied groups), and coronal sections (10 μl) were obtained. Sham control sections (n=2) were also obtained. TUNEL study was performed as our previous report [6]. For immunofluorescent staining, the coronal sections at caudate level were incubated together with 1 : 10 dilution of rabbit anti-cleaved caspase-9 polyclonal antibody (# 9501S, Cell Signaling Tech, MA) and 1 : 40 dilution of mouse anti-active caspase-3 monoclonal antibody (# IMG-144, San Diego, CA) overnight at 4℃. After washing, the sections were incubated with both secondary antibodies: Goat anti-rabbit IgG-FITC (# 5570500, Biomedical Tech Inc, USA) and Goat anti-mouse IgG-TRITC (# 108H9165, Sigma Chemical Co, USA) for 30 min at room temperature. Immunofluorescence was evaluated by a fluorescence microscope with a filter cube (excitation filter, 541 nm; suppression 572 nm) for TRITC labeling and another filter cube (excitation filter, 490 nm; suppression filter, 520 nm) for FITC labeling. For the detection of double immunofluorescence, both cubes were used.

The positive cell stainings were counted in the three 1 × 1 mm^2 regions of inner boundary zone of subsequent infarction or control cerebral cortex. Statistical analyses were performed using Student's t-test for infarct volume, two way analysis of variance (ANOVA) followed by Bonferroni/Dunnett for infarct area and positive cells, or ANOVA repeated measure for rCBF data, respectively.

There was no significant difference in physiological parameters such as pH, pO_2 and pCO_2 between the vehicle- and three GDNF-treated groups before and after MCAO. There was also no significant difference in rCBF between vehicle and GDNF groups similar to our previous report [19]. The infarct volumes of GDNF-treated groups were significantly reduced when GDNF was applied at 0 h (177.0 ± 52.8 mm^3, n=6, mean ± SD; $p<0.01$), and 1 h (220.2 ± 51.5, n=7; $p<0.05$), but not at 3 h (250 ± 57.7, n=6), as compared with vehicle group (295.7 ± 56.6, n=7) (Fig. 1).

TUNEL and caspase stainings were negative in the sham control brain section (Fig. 3a, e, i, m), but became strongly positive in the brain at 12 h of reperfusion with vehicle treatment (Fig. 3b, f, j, n). Positive cells were distributed in the ischemic core and border of occluded MCA area. TUNEL labeling was essentially found in the nucleus of cells. The number of TUNEL positive cells was larger than the positive cells of caspase stainings (Fig. 2). Treatment with GDNF significantly reduced the number of both TUNEL and active caspases -3 and -9 positive cells in the border of the infarcts, especially when GDNF was applied at 0 h (Fig. 2; Fig. 3c, k, o) of reperfusion than 3 h (Fig. 2; Fig. 3d, l, p).

In the double staining, some cells were positive only for active caspase-3 (Fig. 3k, arrowheads) or active caspase-9 (Fig. 3o, arrows), or double positive (Fig. 3f). Treatment with GDNF reduced the number of double positive cells as well (Fig. 3g).

In this study, we demonstrated that topical application of GDNF significantly reduced infarct size in a time-dependant manner. The extent of penetration of

GDNF was roughly 2/3 part of the affected hemisphere which cover the MCAO territory (data not shown). Amelioration of the infarct size by GDNF application was the strongest at 0 h, still effective at 1 h, but became unsignificant at 3 h (Fig. 1). This effect was closely related to the reduction of the numbers of TUNEL, active caspase-3 and -9 positive cells (Fig. 2; Fig. 3). Because there was no significant difference between the vehicle and GDNF groups in the rCBF before, during and after MCAO, the protective effect was not secondary to rCBF improvement but primarily to reducing apoptotic pathways by GDNF. DNA fragmentation detected by TUNEL method can be found in both apoptotic and necrotic neurons, although they can be discriminated morphologically [5]. Larger number of TUNEL-positive cells than active caspase-3 or caspase-9 positive cells (Fig. 2) may be due to colocalization of necrotic and apoptotic cells in the boundary region of infarction.

Caspase-3 is activated through caspase-9 or other pathway [8, 10, 14]. Presence of active caspase-9 negative but active caspase-3 positive cells (Fig. 3k, arrowheads) suggests an activation of the alternative pathway in the ischemic brain [14]. Double positive cells suggest the serial activation of caspase-9 and caspase-3 in the cells (Fig. 3f). Treatment with GDNF reduced the number of TUNEL and active caspase-3 positive cells, while the reduction was weak in active caspase-9 or double positive cells (Fig. 2). Thus, the effect of GDNF was stronger in suppressing active caspase-3 than active caspase-9, or the effect of GDNF may be weak in the pathway of cytochrome c, caspase-9, and caspase-3.

Therapeutic time window of many drugs have been reported after cerebral ischemia. Combinations of an NMDA receptor antagonist (MK-801) and a free radical scavenger (alpha-phenyl-tert-butyl-nitrone, PBN), increased the therapeutic window up to 3 h [4]. Combinations of a GABA agonist and glutamate antagonist showed a synergistic neuroprotective effect up to 4 h following the onset of cerebral ischemia [12]. However, such therapeutic time window has not been reported in neurotrophic factors such as GDNF or others. This study first demonstrated a time-dependant ameliorative effect of GDNF after transient

cerebral ischemia, while the time window was shorter than previous report with other drugs above mentioned. Because the effect of GDNF seemed to be weaker in cytochrome c and caspase-9 pathway, potentiation of this pathway with other drug may further prolong the time window in the future.

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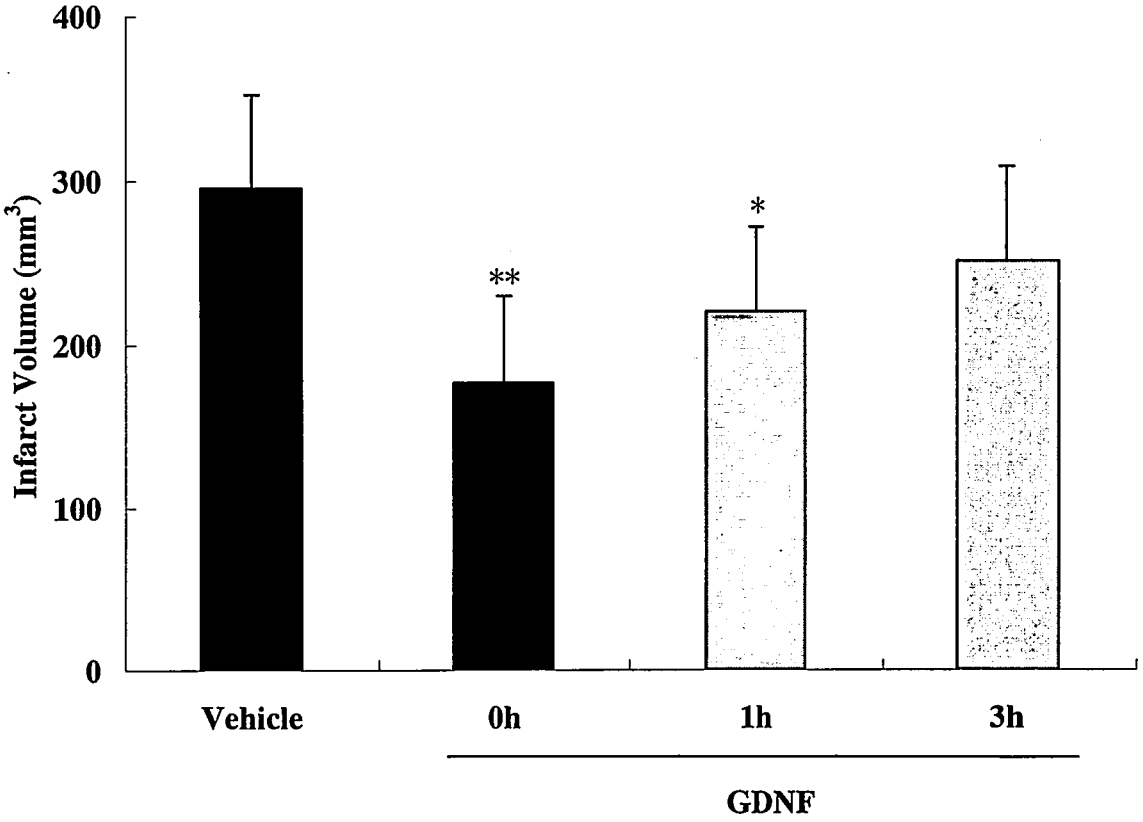
Figure legends

Fig. 1) Effects of GDNF (GDNF 0h, 1h and 3h) on infarct volume at 24 h after 90 min of transient MCAO. Note significant reduction of the GDNF treatment at 0 h and 1 h (**p<0.01, *p<0.05).

Fig. 2) Number of TUNEL and active caspases-3 and -9 positive neurons with or without GDNF at 12 h after transient MCAO. Note significant reduction in the number of both TUNEL and active caspase-3 positive cells in the border of the infarcts at 0 h of treatment (*p<0.05).

Fig. 3) Representative stainings of TUNEL (a-d), double (e-h), active caspase-3 (i-l) and active caspase-9 (m-p) in sham control (a, e, i, m), vehicle (b, f, j, n), GDNF treatment at 0 h (c, g, k, o) and 3 h group, respectively. In contrast to the sham control group (left column), large number of cells became positive in the vehicle group (second column), which are reduced in the GDNF 0 h group especially in TUNEL and active caspase-3 (p<0.05) (third column), less change in GDNF 3 h group. Magnifications, ×200. Bar in panel p, 32 μm.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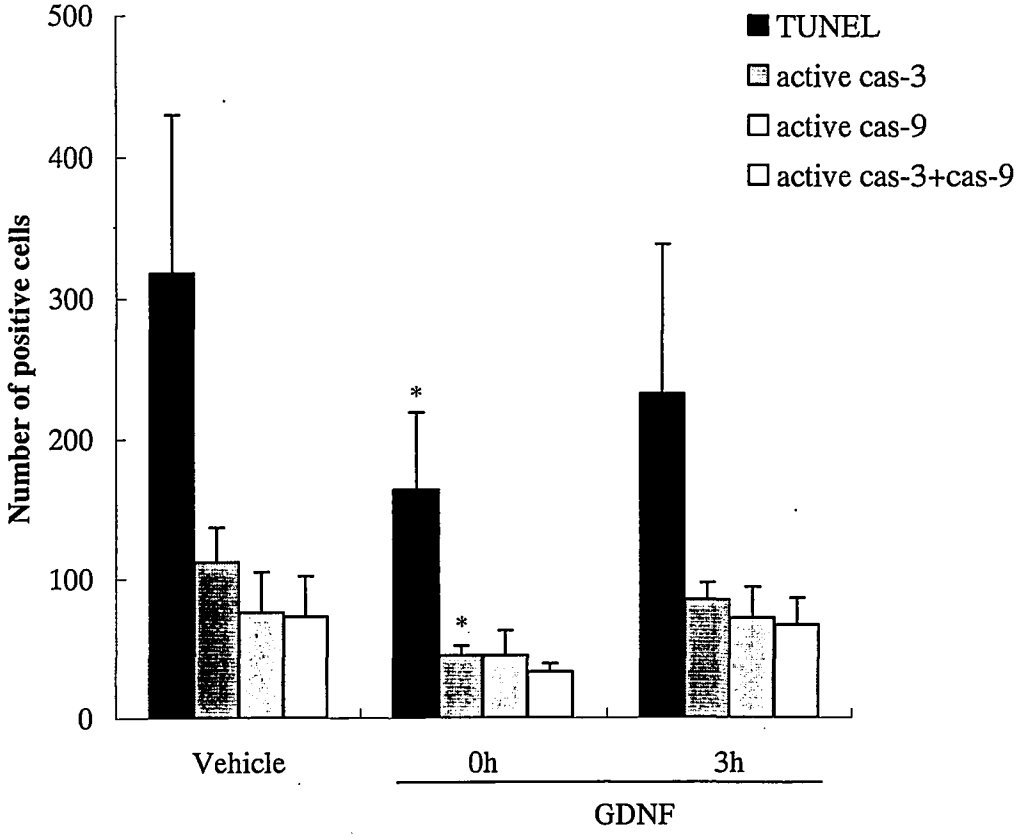
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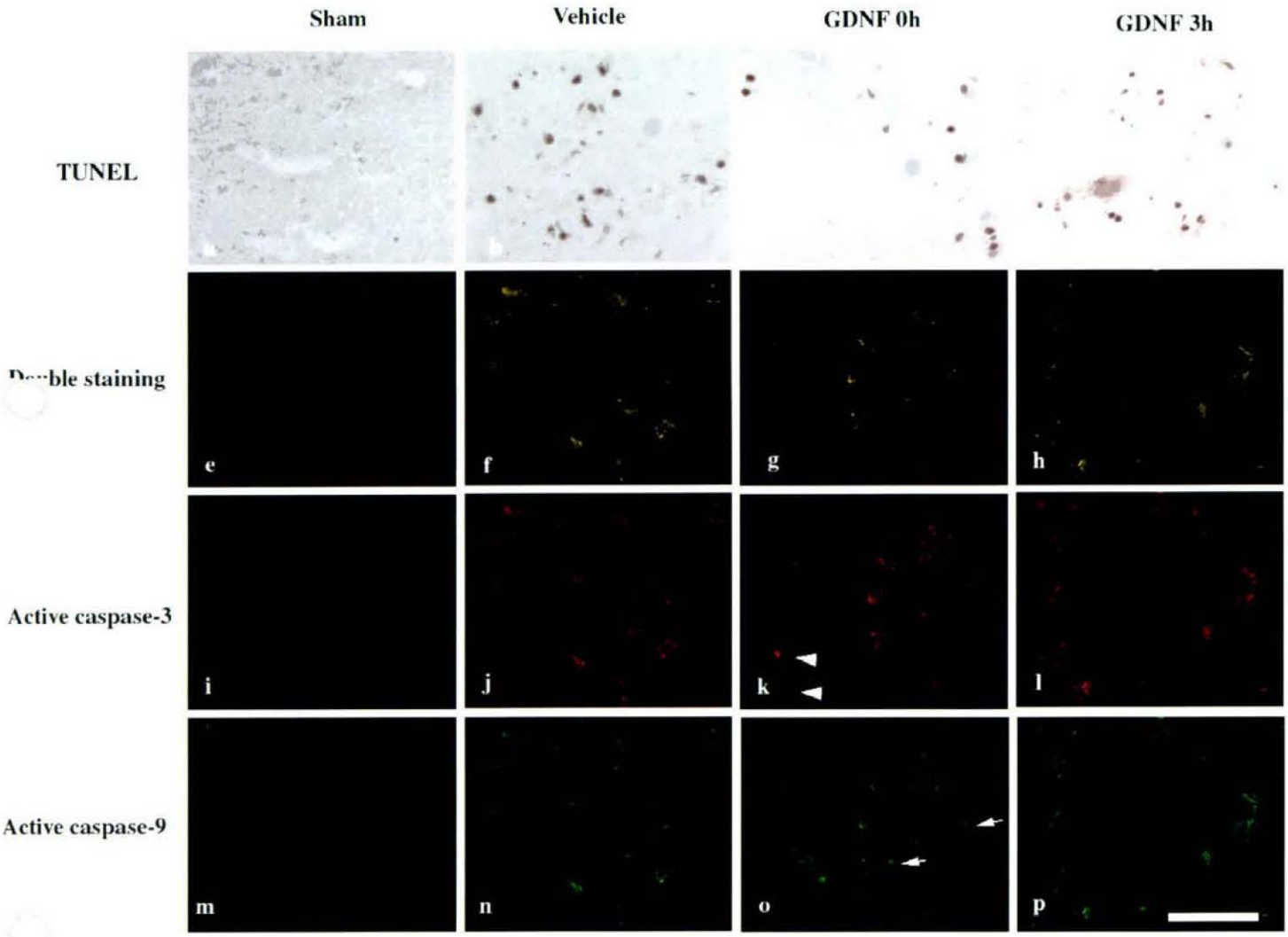
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