Administration of Hematopoietic Cytokines in the Subacute Phase After Cerebral Infarction Is Effective for Functional Recovery Facilitating Proliferation of Intrinsic Neural Stem/Progenitor Cells and Transition of Bone Marrow– Derived Neuronal Cells

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Background—Hematopoietic cytokines, granulocyte colony-stimulating factor (G-CSF), and stem cell factor (SCF) were reported to show a neuroprotective effect or to support neurogenesis. These cytokines also mobilize bone marrow (BM) cells into the brain, and the BM-derived cells differentiate into neuronal cells. We administered these hematopoietic cytokines after focal cerebral ischemia and assessed their effects and the therapeutic time window for neuronal regeneration.

Methods and Results—We induced permanent middle cerebral artery occlusion in mice whose BM had been replaced with BM cells from green fluorescent protein (GFP)-transgenic mice. The occluded mice were treated with G-CSF and SCF in the acute phase (days 1 to 10) or subacute phase (days 11 to 20), and the brain functions and histological changes were evaluated. Separately, we injected bromodeoxyuridine during cytokine treatment to assess cell kinetics in the brain. Six mice were prepared for each experimental group. Administration of G-CSF and SCF in the subacute phase effectively improved not only motor performance but also higher brain function, compared with acute-phase treatment. Acute-phase and subacute-phase treatments identically reduced the infarct volume relative to vehicle treatment. However, subacute-phase treatment significantly induced transition of BM-derived neuronal cells into the peri-infarct area and stimulated proliferation of intrinsic neural stem/progenitor cells in the neuroproliferative zone.

Conclusions—Administration of G-CSF and SCF in the subacute phase after focal cerebral ischemia is effective for functional recovery, enhancing cytokine-induced generation of neuronal cells from both BM-derived cells and intrinsic neural stem/progenitor cells. Because G-CSF and SCF are available for clinical use, these findings suggest a new therapeutic strategy for stroke. (Circulation. 2006;113:701-710.)

Key Words: cerebral infarction ■ regeneration ■ bone marrow ■ hematopoietic cytokines ■ neural stem/progenitor cells

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In mammalian animals, neurogenesis used to be thought to be completed during embryonic life. During the last decade, however, it has been found that neural stem cells (NSCs) exist, neurogenesis continues into adult life, and neuronal cells can be generated in response to insults in adult brain. Because various cytokines appear to play a role in the neurogenesis and/or neuronal regeneration, cytokine treatments may provide a new therapeutic strategy for neurological diseases. Interestingly, several major hematopoietic cytokines also influence the proliferation of neural precursor cells; it was reported that NSCs have c-kit, which is the receptor for one of the major hematopoietic cytokines, stem cell factor (SCF), and SCF stimulates neurogenesis in vivo. Neural cells were also reported to have the receptor for the other hematopoietic cytokine, granulocyte colony-stimulating factor (G-CSF), and intravenous administration of...
G-CSF achieved a significant neuroprotective effect after focal cerebral ischemia. On the other hand, G-CSF and SCF are also well known to effectively mobilize hematopoietic stem cells (HSCs) into the peripheral blood in animal models and also in clinical settings. It was recently reported that bone marrow (BM)-derived cells migrate into the brain with or without cytokine mobilization and give rise to neurons in rodents and humans.

Thus, we assumed that in vivo administration of these hematopoietic cytokines after stroke would facilitate neuronal regeneration by both intrinsic NSCs and BM-derived cells. However, it remained unclear how these different cells contribute to the hematopoietic cytokine-induced regeneration and when is the most appropriate time to administer the cytokines for facilitating neurogenesis. To address these questions, we induced focal cerebral ischemia in mice whose BM cells had been genetically marked; we then injected SCF and G-CSF and assessed the clinical effects and contributions of intrinsic NSCs and BM-derived cells to the generation of new neurons. Our results demonstrated that the cytokine treatment in the subacute phase after ischemia effectively improves brain functions and promotes generation of neuronal cells by facilitating proliferation of intrinsic neural stem/progenitor cells and transition of BM-derived neuronal cells into the brain.

Methods

Mice
C57BL/6J mice were purchased from Japan CLEA (Tokyo, Japan). Green fluorescent protein (GFP)-transgenic mice (C57BL/6 background) were provided by Prof Okabe (Osaka University, Osaka, Japan). All experimental procedures and protocols were approved by the Animal Care and Use Committee of Tokai University School of Medicine. Six mice were prepared for each experimental group.

BM Transplantation
Ten- to 12-week-old GFP-transgenic mice were used as BM donors. Eight- to 10-week-old C57BL/6 mice were used as recipients of the BM cells. Recipient mice were administered a single 850-cGy dose of whole body irradiation. Five million unfractionated BM cells were harvested from femora and tibiae of the donor mice and injected into the tail vein of the irradiated recipients. For analysis of engraftment of donor BM cells, peripheral blood was obtained from the retro-orbital plexus of the recipient mice through the use of heparin-coated micropipettes (Drummond Scientific Co). Red blood cells were lysed by a hemolytic solution containing 0.16 mol/L NH₄Cl. Cells derived from donor GFP mice were detected directly on a FACS Calibur (Becton Dickinson). Eight weeks after BM transplantation, chimerism of GFP+ cells in the recipients’ peripheral blood was 94±3% (mean±SD).

Induction of Focal Cerebral Ischemia
Anesthesia was induced in mice with 4% isoflurane/66% N₂O/30% O₂ and maintained with 1.5% isoflurane. Permanent focal ischemia was achieved as follows: a 2-mm hole was drilled at a site superior and lateral to the left foramen ovale to expose the left middle cerebral artery. The proximal portion of the left middle cerebral artery (MCA) was permanently occluded over a 1-mm segment distal to the origin of the lenticulostriate branches through the use of a bipolar coagulator.

Cytokine Treatment
Twenty-four hours after MCA occlusion, the mice were subcutaneously injected with 100 µL of saline solution once per day for 20 consecutive days. During the first (days 1 to 10) or last 10 days (days 11 to 20) after MCA occlusion. Figure 1. Experimental protocol 1. Five million GFP+ BM cells were transplanted into lethally irradiated recipient mice. Eight weeks later, focal cerebral ischemia was induced in the recipients by MCA occlusion. BM cells were then mobilized into infarcted brain by administration of G-CSF and SCF. Control mice (no-cytokine group) received only saline solution for 20 days from day 1 after MCA occlusion. In cytokine-treated mice, two cytokines were dissolved in the saline solution and administered through days 1 to 10 (group A) or days 11 to 20 (group B) after MCA occlusion. Four weeks after occlusion, brain functions were analyzed. The mice were then killed and their brains were histochemically assessed.

Figure 2. Neurological behavioral measurement. a, Results of the rotor rod test. Falling latency was measured in each group (n=6); *P<0.05, **P<0.01. b, Results of the Morris water maze. The time to reach the platform was measured in each group (n=6); *P<0.05, **P<0.001.
G-CSF and SCF has a synergistic effect on BM cell mobilization.27

present study because it is well known that the combination of
these two cytokines in some experimental groups of mice in the
unpublished data, respectively). We examined the combination of
mobilization of BM cells into the circulation (previous report25 and
dosage regimens of G-CSF alone and SCF alone generate sufficient
solution and injected into the mice; we have confirmed that these

Figure 3. Estimation of infarct volume and immunohistochemical analysis of infarcted area. a through c, Representative morphologies at +0.48 mm from the bregma of the infarcted brain, stained with hematoxylin and eosin; a, Vehicle-treated (no-cytokine) group; b, group A; and c, group B. Bars indicate 200 μm. d, Percentage infarct volume of each group (n=6). Compared with the vehicle-treated (no-cytokine) group, administration of G-CSF and SCF significantly reduced the percentage infarct volume. There was no difference between groups A and B; *P<0.05. e, Low-magnification view of the infarcted brain at +0.70 mm from the bregma. A representative finding of group B is shown. Arrows indicate infarcted areas where numerous GFP⁺ cells were accumulated. Green and blue signals represent GFP and TOTO-3, respectively. Bar indicates 1000 μm. f, High-magnification view of the infarcted area. A representative finding of group B is shown. Most GFP⁺ cells in the infarcted area were round in shape and CD45⁺, indicating that they were infiltrative hematopoietic cells. Green, red, and blue signals represent GFP, CD45, and TOTO-3, respectively. Bars indicate 20 μm.

Neurological Behavioral Measurement
Mice were subjected to behavioral tests 4 weeks after MCA occlusion. The tests measured (1) coordination/balance with the rotor rod test and (2) spatial learning/memory with the Morris water maze.28

Rotor Rod Test
The mice were placed on a drum (diameter, 5.8 cm) that was turned at a speed of 13 rpm, at which all the control mice could maintain position for 120 seconds. If the experimental mice fell within 120 seconds, the latency was recorded. If the mice maintained their position for 120 seconds, a time of 120 seconds was assigned. The latency to escape onto the hidden platform during each trial and were allowed to rest on the platform for 30 seconds after they found it. Mice that failed to find the platform within the allotted period were placed on it by the experimenter, and a time of 120 seconds was assigned. The latency to escape onto the hidden platform in the last trial was adopted. Normal mice reached the platform within 10 seconds.

Bromodeoxyuridine Incorporation Assay
We used bromodeoxyuridine (BrdU) (Sigma Aldrich) to label proliferating cells in the brain. Mice received BrdU (50 mg/kg per day, sc) for 2 days, and 24 hours after the last injection, the brain tissues were fixed as described below.

Estimation of Infarct Volume
Brain specimens were picked up from serial specimens at 2-mm intervals starting at 3 mm from the frontal tip. The specimens were then stained with hematoxylin and eosin and photographed with the use of a digital camera. In each mouse, the volume of infarct in the cerebral cortex was determined by the method of Swanson et al.29 using NIH Image (National Institutes of Health). The volume of noninfarcted cortex was then calculated by multiplying each area by the distance between sections. The infarct volume was determined as

11 to 20), 300 μg/kg of recombinant human G-CSF and/or 100 μg/kg of recombinant murine SCF was dissolved in the saline solution and injected into the mice; we have confirmed that these dosage regimens of G-CSF alone and SCF alone generate sufficient mobilization of BM cells into the circulation (previous report25 and unpublished data, respectively). We examined the combination of these two cytokines in some experimental groups of mice in the present study because it is well known that the combination of G-CSF and SCF has a synergistic effect on BM cell mobilization.27 Control mice received the saline solution without G-CSF and SCF for 20 days.
the percentage difference between the left and right volumes normalized to the volume of the structure in the control cortex. The measurement of infarct size was carried out with the examiners blinded as to the animal’s experimental status.

**Immunofluorescence Photography**

Cryostat sections (8 μm thick) were stained overnight at 4°C with the use of specific antibodies. Rabbit polyclonal anti-GFP (MBL) or mouse monoclonal anti-GFP (Molecular Probes), mouse monoclonal anti-neuronal nuclear antigen (Neu-N; Chemicon), rabbit polyclonal anti-GFAP (Dako), rabbit polyclonal anti-NG2 (Chemicon), rat monoclonal anti-Musashi-1,30,31 rat monoclonal anti-F4/80 (Sero-tec), rat monoclonal anti-CD45 (BD Pharmingen), and rat monoclonal anti-CD31 (BD Pharmingen) were used to identify GFP⁺ cells, neuronal cells, astrocytes, oligodendrocytes, NSCs, microglia, hematopoietic cells, and endothelial cells, respectively, using the methods recommended by the suppliers or reported in the cited references. In the BrdU incorporation assay, sections were reacted with a specific antibody of the BrdU In-Situ Detection Kit (BD Pharmingen), according to the manufacturer’s instruction. The sections were then incubated with secondary antibodies conjugated with Alexa 488, 568, or 594 (Molecular Probes). Nuclei were stained with
TOTO-3 (Molecular Probes). Slides were observed under a confocal laser scanning microscope (LSM 510; Carl Zeiss).

Statistics
Values are presented as mean±SD. The significance of differences among mean values was determined by 1-factor or 2-factor factorial ANOVA, as appropriate. Post hoc analyses were performed, and the Bonferroni/Dunn rule was applied to control for the inflation in type I error. The criterion of significance was $P<0.05$.

Results
We first examined whether administration of G-CSF and SCF affects brain functions after MCA occlusion and whether the mobilized BM cells play a role in the regeneration of infarcted brain tissue. According to the experimental protocol...
shown in Figure 1, BM cells separated from GFP-transgenic mice were transplanted into lethally irradiated mice. Eight weeks after BM transplantation, we induced MCA occlusion into the chimeric mice and injected G-CSF and SCF for 10 days in the acute phase (days 1 to 10 after MCA occlusion; group A) or in the subacute phase (days 11 to 20 after MCA occlusion; group B). Four weeks after occlusion, we assessed the brain functions of the infarcted mice. In the rotor rod test, falling latency was significantly longer in cytokine-treated groups, especially in group B, than in the no-cytokine group (Figure 2a). In the Morris water maze, the time to reach the platform was significantly shorter in cytokine-treated groups than in the no-cytokine group (Figure 2b). All mice of group B reached the platform within 40 seconds, whereas no mice reached the goal within 120 seconds in the no-cytokine group. These results indicated that administration of G-CSF and SCF after occlusion, especially in the subacute phase, effectively improved motor performance and cognitive brain function.

We next killed these experimental mice and analyzed the brain. The percentage infarct volumes of the cytokine-treated groups were identical and significantly lower than that of the no-cytokine group (Figure 3, a through d). We then immunohistochemically analyzed the brain tissues. Low-magnification fluorescence photographs showed strong GFP signals in the infarcted area of each group (Figure 3e and data not shown). In each group, high-magnification photographs taken after triple immunostaining revealed that most of the GFP+ cells in the infarcted area were round or oval in shape and were positive for CD45, indicating they were infiltrative hematopoietic cells (Figure 3f and data not shown). On the other hand, in the peri-infarct area, a number of ramified GFP+ cells were observed (Figure 4, a and b, and data not shown). The number of GFP+ cells in the peri-infarct area was significantly increased by cytokine treatment compared with the no-cytokine group (Figure 4c). Most of them were negative for GFAP and NG2 but positive for F4/80, indicating that they were microglia (Figure 4d). In addition, GFP+ perivascular cells were also frequently observed; they were located on the abluminal side of the endothelial cells and were negative for the endothelial cell marker CD31 (Figure 4e). Moreover, in cytokine-treated groups, especially in group B, we found that some GFP+ cells in the peri-infarct area showed stellate, ramified, or triangular morphologies, and the nuclei of these cells were positive for Neu-N, indicating that they were BM-derived neuronal cells (Figure 5, a through e). We rarely observed BM-derived neuronal cells without cytokine treatment, as reported previously (Figure 5e). We could not find any GFP+GFAP+ cells or GFP+NG2+ cells, that is, BM-derived macroglia, in the infarcted brain.

We next examined the BrdU incorporation assay to analyze the cell kinetics in the infarcted brain tissue. According to the experimental protocol shown in Figure 6, we induced focal cerebral ischemia at 8 weeks after transplantation of GFP+ BM cells by means of MCA occlusion and injected the infarcted chimeric mice with G-CSF, SCF, or the combination of G-CSF and SCF from day 11 after MCA occlusion for 5 days. On days 14 and 15, BrdU was also injected into these mice. After the last cytokine injection, we killed the mice and histochemically analyzed the infarcted brain tissues. Confocal laser scanning microscopy revealed that the number of BrdU+ cells in the infarcted hemisphere was markedly increased in both G-CSF–treated and SCF–treated mice compared with that in the no-cytokine group (Figure 7a). The combination of the two cytokines further increased the number of BrdU+ cells. Most of the BrdU+ cells in the infarcted or peri-infarct area were GFP+ perivascular cells, GFP+NG2+ cells, GFP+GFAP+ cells, or GFP+NG-2+ cells (Figure 7, b and c, and data not shown). However, BrdU+ GFP+ Neu-N+ cells were never found, indicating that G-CSF and SCF mobilized BM-derived neuronal cells into the brain but did not facilitate their proliferation in the infarcted brain. On the other hand, both G-CSF and SCF increased the number of BrdU+ cells coexpressing the marker for neural stem/progenitor cells, Musashi-1,30,31 in the rostral subventricular zone (Figure 7d). The combination of two cytokines further increased the number. However, those BrdU+Musashi-1+ cells were negative for GFP (Figure 7e), indicating that they are neural stem/progenitor cells intrinsic to the brain but not bone marrow–derived cells. G-CSF and SCF also increased the number of BrdU+Musashi-1+GFP+ cells in the subgranular layer of the dentate gyrus (data not shown), though these cells were not detected in either the infarcted or the peri-infarct area. We further compared the numbers of BrdU+ cells and BrdU+Musashi-1+ GFP+ cells in the subacute phase with those in the acute phase; for the analysis of the acute phase, mice were injected G-CSF/SCF or saline during days 1 through 5 and BrdU on days 4 and 5 and killed on day 6 after cerebral infarction. The numbers of BrdU+ cells in the infarcted hemisphere (Figure 8a) and of BrdU+Musashi-1+GFP+ cells in the rostral subventricular zone facing the lateral ventricle (Figure 8b) in the subacute phase were significantly higher than those in the acute phase, presumably reflecting the
response to the cytokines. Collectively, these results indicated that G-CSF and SCF exerted trophic effects in the subacute phase by synergistically facilitating the proliferation of intrinsic neural stem/progenitor cells in the neurogenic sites.

Discussion

It was recently found that neuronal cells have receptors for both SCF and G-CSF, and in ischemic animal models, in vivo administration of SCF stimulated proliferation of immature neurons, whereas that of G-CSF improved motor performance and decreased the size of the infarcted area, showing a neuroprotective effect. On the other hand, it was reported that administration of G-CSF and SCF increased the number of BM-derived Neu-N$^+$ cells in nonischemic brain of mice. In this study, we examined administration of G-CSF and SCF in both the acute phase and subacute phase after focal cerebral ischemia and found that the subacute phase treatment effectively improved not only motor performance but also higher brain function, compared with the acute phase treatment. The acute phase and subacute phase treatments identically reduced the infarct volume. Nevertheless, this reduction is unlikely to be associated with a neuroprotective effect, because neuronal death is known to be completed within a very early period after ischemia. We speculated that the reduction of infarct volume observed in the cytokine-treated mice might be associated with not only neuronal regeneration but also enhanced scavenging of lipid peroxide–bearing damaged cells by an increased number of cytokine-
mobilized macrophages and microglia. However, the subacute phase treatment significantly induced neuronal regeneration by BM-derived cells in the peri-infarct area and stimulated proliferation of intrinsic neural stem/progenitor cells in the neuroproliferative zone, compared with the acute phase treatment. Thus, it was concluded that hematopoietic cytokine treatment in the subacute phase after focal cerebral ischemia favors neuronal regeneration, and this is reflected in better functional recovery. In general, in the acute phase, severe inflammation occurs, and many inflammatory cytokines and enzymes such as tumor necrosis factor-α, interleukin-1, cyclooxygenase 2, matrix metalloproteinase, and caspases, which have neurotoxic effects, are increased in the brain tissue. On the other hand, at the subacute phase, the acute inflammation is over, and the microenvironment is likely to have changed to the reparative or remodeling phase, with increased levels of various growth and neurotrophic cytokines. Then, G-CSF and SCF might effectively induce neurogenesis in cooperation with other neurotrophic cytokines in the present study. Our preliminary studies of the levels of cytokines within the ischemic brain have indicated that messenger RNA expression of several anti-inflammatory cytokines, such as interleukin-10, is increased in the subacute phase in the cytokine-treated mice compared with the vehicle-treated mice (data not shown). Interestingly, the subacute phase was also reported to be suitable for neuronal regeneration in a spinal cord injury model; transplantation of neural progenitor cells into the injury site on day 9 after spinal cord contusion injury resulted in significant neurogenesis and functional recovery in adult rats, whereas almost none of the transplanted cells survived when neuronal progenitor cells were transplanted 24 hours after the injury.

Several reports have demonstrated that BM-derived cells are capable of differentiating neuronal cells in vivo without cell-fusion events in rodents and humans, and the origin of the BM-derived neuronal cells was suggested to be HSCs. On the other hand, other investigators described that BM stromal cells could also differentiate into neuronal cells in vitro and in vivo. Therefore, we tried to determine whether mesenchymal stem cells (MSCs) or MSC-derived cells can be mobilized and differentiate into neuronal cells in our experimental system with mice whose BM showed engraftment of MSCs derived from a GFP-labeled clonal cell line, CMG. However, there were no GFP+ cells in the infarcted brain (unpublished observation). Further studies using naive MSCs will be necessary to determine the true potential of BM MSCs, although it has been impossible to isolate pure MSCs from crude BM cells using cell-surface phenotypes so far.

We established that the mobilized BM-derived cells found in the infarcted brain do have, or have already lost, stem/progenitor cell activities, because we never observed glial marker–positive BM-derived cells or proliferative activity, that is, BrdU uptake, of BM-derived neuronal cells in the present study. The hematopoietic cytokine treatment appeared to induce “transition” of BM-derived neuronal cells to the peri-infarct area. Interestingly, a distinctive population of hematopoietic cells expressing neural genes was very recently found in murine BM; the genes could be regulated in the infarcted left hemisphere was counted for each group (n=6), using the specimen at −1.30 mm from the bregma. Two-factor factorial ANOVA revealed significant effects: effect of treatment phases on the number of BrdU+ cells, interaction, P=0.0002; effect of G-CSF/SCF on the number of BrdU+ cells, P=0.0001; interaction, P=0.0001. **P=0.0001. The number of BrdU+ Musashi-1+ cells in the subventricular zone was counted for each group (n=6), using the specimen at −1.00 mm from the bregma. Two-factor factorial ANOVA revealed significant effects: effect of treatment phases on the number of BrdU+ Musashi-1+ cells, P=0.0001; effect of G-CSF/SCF/NS; **P=0.0001. }

**Figure 8.** Comparison of the number of BrdU+ cells between the acute and subacute phases. G-CSF and SCF (G-CSF + SCF) or saline (no cytokine) were injected into mice from day 1 (acute phase) or from day 11 (subacute phase) for 5 days after cerebral infarction. BrdU were also injected into all mice on the last 2 days of cytokine or saline administration, and the mice were then killed. a, The number of BrdU+ cells in the infarcted left hemisphere was counted for each group (n=6), using the specimen at −1.30 mm from the bregma. Two-factor factorial ANOVA revealed significant effects: effect of treatment phases on the number of BrdU+ cells, P=0.0001; effect of G-CSF/SCF on the number of BrdU+ cells, P=0.0001; interaction, P=0.0002; **P=0.0001; NS; **P=0.0001.
observations of proliferation of intrinsic neural stem/progenitor cells at these neurogenic sites after ischemia. Avrdsen et al recently reported that new neurons generated in the subventricular zone and dentate gyrus after stroke migrate into the damaged area.

The cytokine treatment also stimulated marked transition of BM-derived microglial cells and perivascular cells. These two types of cells were reported to have originated from HSCs and to support brain tissue repair by releasing neurotrophic cytokines and by playing a crucial role in vessel stabilization and control of angiogenesis, respectively. Therefore, these HSC-derived cells would also contribute in a part to the functional recovery in the cytokine-treated mice. Thus, we will further assess the microenvironment of the infarcted area to examine more precisely the mechanisms and potential of cytokine therapy for cerebral ischemia.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Stroke is a leading cause of death and disability worldwide; therefore, a new therapeutic strategy for stroke is needed. Recently, it has been found that neuronal cells can be generated in response to insults in adult brain and, interestingly, several hematopoietic cytokines influence the proliferation of neural precursor cells. On the other hand, it has also been reported that BM-derived cells migrate into the brain with hematopoietic cytokine mobilization and give rise to neurons there. Thus, in this study, we examined a therapy with two clinically available hematopoietic cytokines, that is, G-CSF and SCF, in an animal stroke model and assessed when is the most appropriate time to administer the cytokines and how intrinsic neural cells and BM-derived cells contribute to the neuronal regeneration in vivo. As a result, administration of G-CSF and SCF in the subacute phase (day 11 to 20 after stroke) effectively improved not only motor performance but also higher brain function, compared with acute phase (days 1 to 10 after stroke) treatment. Furthermore, subacute phase treatment significantly induced transition of genetically identifiable BM-derived neuronal cells into the peri-infarct area and stimulated proliferation of intrinsic neural stem/progenitor cells in the neuroproliferative zone. Our results suggest a potential clinical application of subacute phase cytokine treatment for stroke.
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