Collateral arteries grow from preexisting anastomoses in the rat hindlimb

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Collateral arteries grow from preexisting anastomoses in the rat hindlimb. Am J Physiol Heart Circ Physiol 283: H2012–H2020, 2002. First published July 18, 2002; 10.1152/ajpheart.00257.2002.—Previous findings have suggested that collateral arteries grow from preexisting arteriolar anastomoses (“arteriogenesis”). To investigate whether collateral growth occurs without preceding angiogenesis, we obtained vascular casts and postmortem angiographies at 3, 7, and 21 days after unilateral femoral artery occlusion in the rat. Proliferation kinetics were determined after 5′-bromo-2′-deoxyuridine infusion. A preexisting anastomosis was identified. Proliferation of this vessel began 24 h after femoral artery occlusion, increased maximally during the first 3 days, and reached 60% at day 7. Cell division was restricted to preexisting anastomoses and occurred neither in directly neighboring arterial vessels nor in capillaries. Collateral vessels doubled their diameter within 7 days and assumed a typical corkscrew appearance (increase of length: 21%). After 7 days of occlusion, we measured a further increase of length (14%) but no proliferation or increase of diameter. We conclude that arteriogenesis is a biphasic process involving rapid proliferation of preexisting arteriolar shunts followed by pronounced remodeling processes. Arteriogenesis occurs independently of angiogenesis and denotes a separate entity of vascular proliferation.

angiogenesis; arteriogenesis; collateral growth; vascular remodeling

MECHANISMS AND STIMULI leading to collateral growth have been subject of intense debate during the past decade (16, 26, 31). The dispute has been difficult to solve because the growth of a complex structure like that of a collateral artery has evaded direct observations. Angiogenesis, the sprouting of capillaries, in contrast, can be studied in in vitro assays, in chick allantois membrane assays, or in implanted tumors via intravital microscopy. Most adaptive mechanisms in the adult are a recapitulation of embryonal development. In the embryo, a sequence of events has been described leading from vasculogenesis, the in situ accumulation and differentiation of endothelial precursor cells into primary vascular structures, and subsequent angiogenesis, the sprouting of capillaries from preexisting vessels, to the formation of vascular networks. Shortly before birth, this primary network undergoes profound remodeling processes, including invasion of smooth muscle cells as well as regression of other vascular structures that result in the formation of distinct vascular provinces (25). Hypoxia was identified as a major stimulus for angiogenesis during embryonal development as well as during tumor growth (13). Several growth factors and receptors responsible for the different stages of vascular development as well as mechanisms for their induction have been described (31). The most important growth factors originate from the VEGF and FGF family. The angiopoietins appear to be responsible for the necessary assembly and disassembly of vascular structures, and the ephrins appear to be responsible for growth guidance (4, 5). Little, however, is known about the final remodeling step that leads to formation of a mature vasculature. The notion that collateral growth is a recapitulation of the whole process of vascular development in the embryo or at least of hypoxia-driven angiogenesis, followed by subsequent remodeling processes has been challenged by several observations. One of these observations was that collateral arteries grow largely outside the ischemic area at risk and that growth continues beyond the time of peripheral tissue ischemia (14). Fulton (9) had already demonstrated this for the human heart in 1965 merely by analysis of the vascular architecture. We were able to show that larger parts of collateral arteries were growing outside territories with perfusion deficits in a rabbit model of femoral artery occlusion (14). As discussed numerous times, it is difficult to explain why collateral growth occurs just outside the ischemic territory and not within this region if ischemia was the main stimulus. It also was demonstrated that neither the expression of hypoxia-sensitive genes nor the content of ADP, ATP, or AMP changed within the region of collateral growth, indicating that indeed hypoxia is not present in areas in which collateral arteries are growing (7). Another observation challenging the angiogenesis hypothesis was the rapidity at...
which collateral arteries grow. In 1981, Rentrop et al. (22) demonstrated, with the help of consecutive angiographies after myocardial infarction, that 30% of all patients had a recruitable collateral circulation at the time of coronary artery occlusion. In just 2 wk, 90% of all patients developed recruitable collateral arteries (22). Our own data from animal experiments are on the same line. In the dog heart, collateral arteries develop ~4 wk after placement of an Aneurin constrictr. Because we know that constrictr placement leads to a hemodynamic relevant stenosis within 2 wk, we again obtained a period of 2 wk, which is necessary for a collateral circulation to develop within the dog heart (24). In our rabbit hindlimb model, the main rise in collateral conductance occurred within the first week after femoral artery occlusion (14). Although not quantified, proliferation appeared to be maximal during the first 3 days after femoral artery occlusion (2). It is difficult to imagine that arterial vessels with a length of several centimeters carrying several layers of smooth muscle cells grow within 2 wk from sprouting capillaries. To explain these findings, we therefore developed the hypothesis that these collateral vessels grow from preexisting arteriolar anastomoses. Upon occlusion of the main nurturing vessel, the major part of the blood flow is diverted through these small pre-existing vascular connection, which leads to a dramatic increase in flow velocity in these vessels and therefore to a dramatic rise in shear force. This increase in shear force we believed to be the main stimulus for collateral growth. We named this proposed mechanism “recapitulated arteriogenesis.” However, to date, it has not been demonstrated beyond a doubt that it is a preexisting anastomosis that gives rise to a collateral artery. Therefore, our concept has often been misinterpreted in the sense that arteriogenesis merely describes remodeling processes that transform angiogenic blood vessels into arterial vessels (16). This interpretation ascribes adult angiogenesis as the primary mechanism responsible for collateral formation.

In this paper, we demonstrate for the first time that collateral growth can occur without preceding adult angiogenesis. We were able to clearly identify a preexisting arteriolar shunt connecting the internal iliac artery to the popliteal artery in the rat hindlimb. Upon femoral artery occlusion, this vessel starts to proliferate within 24 h and becomes a collateral artery. Vessels of similar size and architecture in the direct vicinity of this vessel that were not part of preformed anastomoses did not proliferate. We were thus able to demonstrate at the level of a single vessel that arteriogenesis occurs independently of angiogenesis and denotes a separate entity of adult vascular proliferation. The ability to follow vascular growth in a single vessel allowed us to analyze the proliferation and growth kinetics for collateral vessels and enabled us to study the early events in arteriogenesis preceding vascular proliferation. This is not possible in models in which the vessels bound to proliferate cannot be identified before they start to grow.

**MATERIALS AND METHODS**

**Animal model.** The present study was performed according to Section 8 of the German Law for the Protection of Animals. Experiments were performed on male Sprague-Dawley rats weighing 400–500 g. Anesthesia was induced by ether inhalation and continued by intraperitoneal injections of ketamine (100 mg/kg body wt, Astra) and 2% xylazine (5 mg/kg body wt, Bayer). The right femoral artery was prepared carefully without damaging the vein and nerve and ligated with two sutures (Resolon 5/0, Resorba) ~1 cm apart, the first one just distal to the artery femoralis profunda. The wounds were closed, and the animal was allowed to recover. Animals designated for proliferation studies were supplied with osmotic minipumps filled with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU; Sigma). Osmotic minipumps (model 2MH1, Alzet, 10 ml/h, 7 days) were filled with 62 mg BrdU dissolved in 3 ml of 0.5 M NaHCO 3 buffer (pH 9.8) under sterile conditions. They were implanted subcutaneously in the neck region.

**Corrosion casting.** Corrosion casting was performed as previously described (11). Rats were anesthetized and anticoagulated (700 units heparin/kg ip). The abdominal cavity was opened, and the aorta was cannulated. Blood was flushed from the organ with saline (37°C at 80–100 mmHg). Resin (Mercox/catalyst, 10/0.3, Ladd Research Industries; Burlington, VT) was infused through the same cannula until the onset of polymerization. The resin-filled tissue was immersed in hot water (50°C) for 1 h to complete resin curing. The tissue was removed by maceration in alternating rinses of 5% KOH and hot water. After the resulting casts were cleaned in distilled water, the arterial tree, including the collateral arteries were carefully stripped from the capillaries and venous tissue for better visualization. Corrosion castings were obtained from five animals at 2 wk and 2 mo after unilateral femoral artery occlusion.

**Postmortem angiography.** Postmortem angiographies were obtained as previously described (14). Gelatin (12 g, type A, from porcine skin, Sigma) was dissolved in 100 ml of heated distilled water under continuous steering. Afterward, 60 g barium sulfate (Merck) was added. Animals were anesthetized again and anticoagulated, and the aorta was cannulated. Subsequently, the animals were bled and immersed in water warmed to 37°C. After the lower part of the body was rinsed with saline (37°C at 80–100 mmHg), the contrast medium was infused with a pressure of 150–180 mmHg until filling of the distal femoral stump was observed. The animals were immediately placed on crushed ice, and the contrast medium was allowed to harden under continuous pressure. Before the angiographies were obtained, the rats were embedded in gelatin (type A, from porcine skin, Sigma) to fix the animal and to obtain an equilibrated thickness for X-ray penetration. X-ray pictures were taken in a X-ray chamber (model 43855D, Faxitron X-Ray) with the use of single paper-wrapped films (X-OMAT MA 13 × 18 cm, Kodak) exposed to 30 kV for 6 min. Pictures were taken at two different angles to allow for stereoscopic analysis of vessel architecture. Identification and counting of collateral arteries was performed with help of a stereoscope (Topecon; Tokyo, Japan). This allowed three-dimensional identification of the stem, midzone, and reentry regions of collateral vessels. Angiographies were obtained from five animals after 7 days of unilateral occlusion and three animals after 21 days of unilateral femoral artery occlusion.

**Determination of diameter, length, and number of collateral arteries.** Only vessels clearly identified as collateral vessels by virtue of a stem, midzone, and reentry region were
counted as collateral vessels. Counting was performed by a blinded observer. Collateral vessel diameter and length were obtained from angiographies using NIH Image software. Diameter and length were only determined in the main collateral vessel, which was reproducibly identifiable in all animals studied. For normalization of contrast filling, midzone diameter was calculated as the ratio to the diameter of the femoral artery just distal to the occlusion.

**Determination of the proliferative index and proliferation kinetics.** After 24 h, 3 days, 7 days, and 21 days, the respective groups of animals were anesthetized again. The aorta was cannulated, the animals were bled, and contrast medium was infused as described before. After being hardened on crushed ice, the midzone of the identified collateral vessels was quickly removed, including the surrounding tissue, embedded in Tissue Tek (OCT compound, Sakura Finetek) on cork plates, and shock frozen in N-hexane (ICN Biomedicals). Cryostat sections (7 μm thick) were fixed in glycine and ethanol (3:7, pH 2.0) at −20°C over 20 min. Sections were washed in phosphate-buffered saline (PBS) before being blocked with 1% BSA (bovine serum albumin, Fluka) for 30 min. For BrdU staining, we used the BrdU working solution as supplied by the manufacturer. As a secondary antibody, we used a Cy2-conjugated goat anti-mouse IgG (Lot 41110, Jackson ImmunoResearch Laboratories; Dianova, Germany) diluted 1:200 in 1% BSA. Nuclear staining was obtained using 5-Bromo-2′-Deoxyuridine Labeling and Detection Kit 2 (Roche Diagnostics) according to the protocol supplied by the manufacturer. As a secondary antibody, we used a Cy2-conjugated goat anti-mouse IgG (Lot 41110, Jackson ImmunoResearch Laboratories; Dianova, Germany) diluted 1:200 in 1% BSA. Nuclear staining was obtained using a 0.001% propidium iodide solution (P-4170, Sigma). Before analysis under the fluorescent microscope, slides were embedded in Mowiol (Calbiochem) und 1,2-phenylenediamine (PPD; Merck). For counting purposes, pictures were taken from four to five sections of three different midzone segments of the main collateral vessel. The proliferative index was calculated as the number of BrdU-positive nuclei (green fluorescence) to the total number of nuclei (red propidium iodide fluorescence). The proliferative index was determined in three animals after 24 h, five animals after 7 days, and three animals after 21 days of unilateral femoral artery occlusion and continuous BrdU infusion.

**Histology.** To evaluate lumen size and vessel wall structure, histological samples of the main collateral artery were obtained after perfusion fixation 3 days, 7 days, 3 wk, and 4 mo after femoral artery occlusion. For perfusion fixation, the aorta was cannulated under general anesthesia and anticoagulation as described above. The lower part of the body was flushed with saline, followed by infusion of 4% formaldehyde at a constant pressure of 150 mmHg for 20 min as described previously (2). After the lower part of the body was flushed again with saline, contrast medium was infused as described above to identify the collateral artery. Midzone segments of the collateral artery were obtained for cryostat sections as described above as well as for paraffin embedding. Cryostat sections were stained with hematoxillin, and paraffin-embedded tissue sections were subjected to hematoxillin-eosin staining.

**Statistical analysis.** Data are presented as means ± SD. Statistical comparisons between groups were performed with Student’s t-test. Differences among means were considered significant when P < 0.05.

**RESULTS**

**Identification of a preexisting arteriolar anastomosis by corrosion casting.** Corrosion casts of both lower extremities of Sprague-Dawley rats revealed a preexisting arteriolar shunt connecting the internal iliac artery to the popliteal artery with a defined stem, midzone, and reentry region. The anatomy was reproducible in five experiments (Fig. 1). The vessel had only a slight tortuous appearance. Midzone diameter was 140 μm. After 2 wk and 2 mo of femoral artery occlusion, this vessel became the most prominent collateral artery with an extremely tortuous course and a midzone diameter of 300 μm. Localization and anatomy was the same in all casts examined and was identical for both the dormant anastomosis as well as for the fully developed collateral vessels, indicating that in-
Indeed, the main collateral artery grows from this preexisting arteriolar anastomosis.

**Development of the main collateral artery from preexisting anastomosis demonstrated by postmortem angiography.** In the following set of experiments, we created stereoscopic postmortem angiograms before as well as 1 and 3 wk postfemoral artery occlusion for analysis of collateral anatomy and development using computerized imaging systems. Again, the preexisting anastomosis was clearly visible at a reproducible anatomic site in all animals studied and was identified as the vessel becoming the main collateral artery (Fig. 2, A and B). The total number of angiographically visible collateral vessels increased significantly from $2.3 \pm 0.5$ before occlusion to $5.3 \pm 0.5$ after 7 days of occlusion and to $7.3 \pm 0.5$ after 21 days of occlusion (control vs. 7 days: $P < 0.00001$; 7 vs. 21 days: $P < 0.01$; Fig. 3A). The major increase was thus found within the first 7 days after occlusion.

The midzone index (midzone diameter to diameter of the femoral artery distal to the ligature) of the main collateral vessel increased from $0.17 \pm 0.05$ before occlusion to $0.28 \pm 0.06$ after 7 days of occlusion (control vs. 7 days: $P < 0.0003$; 7 vs. 21 days: $P < 0.01$; Fig. 3B). The vessel was identified as the main collateral artery (Fig. 2, A and B). The midzone index (midzone diameter to diameter of the femoral artery distal to the ligature) of the main collateral vessel increased from $0.17 \pm 0.05$ before occlusion to $0.28 \pm 0.06$ after 7 days of occlusion (control vs. 7 days: $P < 0.0003$; 7 vs. 21 days: $P < 0.01$; Fig. 3B). The vessel was identified as the main collateral artery (Fig. 2, A and B).
occlusion to 0.46 ± 0.07 after 7 days of occlusion (P < 0.001; Fig. 3B). Interestingly, the midzone index dropped again to 0.31 ± 0.04 after 21 days of occlusion, indicating regression of the collateral vessel after 7 days of occlusion (P < 0.02; Fig. 3B).

We observed not only an enlargement in diameter of the collateral vessel but also a distension of the length due to an increase in vessel tortuosity. The total length of the collateral artery increased by 21% within 7 days after occlusion and significantly by 39% within 21 days after occlusion (P < 0.02; Fig. 3C).

Proliferation is restricted to preexisting arteriolar anastomosis. Staining of the collateral vessel and surrounding structures for BrdU after continuous subcutaneous infusion of the thymidine analog during the first week after femoral artery occlusion revealed that proliferation was restricted to preexisting arteriolar anastomosis (Fig. 4, A–D). No proliferation was seen in the directly neighboring vessel of similar size and vessel architecture that did not connect the ischemic to the nonischemic territory as revealed by postmortem angiography before the tissue sections were obtained (Fig. 4, A and E–G).

Proliferation kinetics of the collateral artery. Proliferation kinetics of the collateral artery were obtained after continuous BrdU infusion for 1, 3, 7, and 21 days. Positively stained nuclei of endothelial and smooth muscle cells represented the total amount of cells proliferating within the observed period and were related to the total amount of nuclei of the vascular tissue. This allowed the calculation of accumulative proliferation indexes. The first BrdU-positive nuclei were seen 3 days after occlusion, indicating that proliferation started between 24 h and 3 days after occlusion. The main rise of the proliferative index occurred between days 1 and 3 after occlusion reaching 35% at day 3 (Fig. 5). At day 7, the proliferative index had risen to 59%. From the onset of the second week after occlusion, there was no further measurable proliferation. The proliferative index at day 21 after occlusion was even slightly lower than at day 7 after occlusion, in keeping with our angiographic data (24 h vs. 3 days: P < 0.01; 3 vs. 7 days: P < 0.01).

Histology of developing collateral vessels. Hematoxylin-eosin staining of perfusion-fixed tissue revealed pronounced remodeling processes beginning 7 days after occlusion. Remodeling was particularly pronounced between days 7 and 21 after femoral artery occlusion. Within this time, an asymmetrical neointima was formed including several layers of smooth muscle cells and several laminae elasticae internae (Fig. 6, A–F). Within 4 mo after occlusion, vessel wall thickness increased several-fold, whereas lumen diameter of the main collateral vessel in the rat only doubled (Fig. 7, A and B).

DISCUSSION

In this study, we present for the first time convincing evidence that collateral arteries grow from preexisting arteriolar anastomoses without preceding angiogenesis. We were thus able to deliver proof of the hypothesis that a mechanism distinct from angiogenesis and vasculogenesis is responsible for collateral growth in the rat hindlimb. Our findings are supported by recent observations by Terjung et al. (17), which indicate that collateral growth and angiogenesis respond differently to NO depletion. The question remains as to whether our model is a good reflection of the human disease. As
Fig. 4. 5-Bromo-2′-deoxyuridin (BrdU) staining of sections of collateral vessels and control vessels obtained from animals infused with the thymidine analog BrdU. Green staining of nuclei denotes proliferating cells that have incorporated BrdU. Red propidium iodide fluorescence shows the total number of nuclei within the section. Green fluorescent proliferating nuclei are only observed in the collateral vessel (A, large open arrow) but not in directly neighboring vessels (A, small open arrows). B and E: propidium iodide fluorescence of the collateral vessel (B) and the control vessel (E) at higher magnification. C and F: green fluorescence of BrdU-positive cells in the same sections. Note that green fluorescent nuclei of proliferating cells are only visible in the collateral vessel (C). No staining is observed in the control vessel (F). D and G: red propidium iodide and green anti-BrdU fluorescence combined in the collateral vessel (D) and the control vessel (G).
mentioned in the Introduction, previous studies have demonstrated the existence of preexisting intra- and intercoronary anastomoses in virtually every human heart and thus the presence of a substrate for the mechanism of collateral growth described in this paper (3, 9). Furthermore, studies on acute coronary occlusions have shown that collateral arteries grow within 1–2 wk in a majority of patients, suggesting that mechanisms similar to those we observed in the rat hindlimb are responsible for vascular growth after myocardial infarction (22). In contrast to acute coronary occlusion, collateral growth takes several months in patients with progressive arterial occlusive disease (21). The stimulus for vascular growth, however, is only present at times in these patients depending on exercise, blood pressure, and several other factors. It usually resolves before severe myocardial damage can occur. The determination of any reliable growth kinetics for such a dynamic situation is nearly impossible. Our study certainly does not preclude a role for angiogenesis under these circumstances. A vascular network would be created de novo giving rise to small arteriolar anastomoses that need to be remodeled to create collateral arteries. At this stage of collateral development, a situation is encountered similar to the one we described for the rat hindlimb.

It was interesting to note that proliferation was restricted to preexisting arteriolar anastomoses but that it did not involve directly neighboring vessels of comparable size and structure that did not connect the nonischemic to the ischemic territory. This confinement of proliferation to certain vessels indicates that the signaling for collateral growth comes from inside the vessel rather than from the surrounding tissues. Thus hemodynamic forces rather than chemical signals released from the surrounding tissue (for example, those evoked by ischemia) are likely to be the primary stimulus for angiogenesis. Shear force is one of the hemodynamic factors that is altered in a preexisting shunt upon occlusion of the main blood-supplying vessel. Glagov et al. (10) were able to demonstrate in numerous experiments that vessels tend to remodel to maintain a certain level of shear force and tensile force. This general principle may also apply to collateral arteries. In fact, Tuttle et al. (29) were able to show in a recent experiment that the extent of remodeling of collateral vessels in the bowel was proportional to flow velocities and shear forces generated by occlusion of nurturing vessels. Our concept that shear force is the primary stimulus for arteriogenesis was challenged on the grounds that proliferation occurs centripetal from the border of the ischemic zone in the rat kidney (31). Shear force is dependent on flow velocity and the radius to the third power. Thus a centripetal spread of proliferation would be very well explainable by shear force if the radius of the preexisting shunt were smallest at the border of the ischemic organ, which is not unlikely.

Shear force depends on vessels architecture and the kind of flow that is generated. There is a wide variation of flow patterns in corkscrew collateral vessels. Consequently, there will be a diversity of proliferation and remodeling processes along the same collateral vessel if shear force were the main stimulus. This in turn would explain the asymmetry of remodeling processes like neointima formation and the increase in vascular tortuosity.

In this study, we indeed demonstrated an increased in tortuosity reflected in the increase in length of the preexisting collateral vessel. In contrast to proliferation and luminal diameter change, which showed a maximum during the first week after occlusion and then came to a halt, longitudinal distension of the collateral artery continued and reached significant values on day 21 after femoral artery occlusion. These findings indicate that collateral growth is a biphasic process beginning with a rapid proliferative phase followed by extensive remodeling, which has already been suggested by our previous studies in the rabbit hindlimb (2).

In conclusion, collateral growth is a biphasic process. It begins with a very rapid onset of massive proliferation leading to significant outward remodeling within 1 week after occlusion. This proliferation slows down considerably or even comes to a standstill after 1 wk of occlusion and is succeeded by a phase of intense inward remodeling that leads to neointima formation and a pronounced increase of vessel wall diameter. In the remodeling phase, we even may encounter regression of the collateral lumen diameter, as seen in our model.

Although reasoning based on these findings indicates that not a general chemical factor such as ischemia but local acting hemodynamic forces are responsible for collateral growth, we were not able to deliver direct evidence for the hypothesis that shear force is the primary stimulus for arteriogenesis. The ability, however, to identify a collateral artery at any stage after induction as presented in this paper is the prerequisite for modeling of flow patterns along a preexisting arteriolar shunt and their evolvement during collateral growth.

Fig. 5. Proliferation kinetics of the collateral vessel. The main rise in the cumulative proliferative index (numbers of proliferating vascular cell/numbers of total vascular cells) occurs between days 1 and 3 after femoral artery occlusion. There is a further rise in the cumulative proliferative index between days 3 and 7. After day 7, no further proliferation is observed (#P < 0.01 and *P < 0.01; +P < 0.05).
Fig. 6. Cryosections of the collateral artery 3, 7, and 21 days after femoral artery occlusion. The contrast medium-filled vessel is thin walled after 3 days of femoral artery occlusion (A and B). There is some protrusion of the endothelium in the collateral vessel after 7 days of occlusion (C and D, large open arrows). After 21 days of occlusion, an asymmetrical neointima (E and F, solid arrows) has formed with two laminae elasticae internae (E and F, open arrows).

Fig. 7. Sections from paraffin-embedded sections of the perfusion-fixed collateral vessel (A) and control vessel (B) after 4 mo of femoral artery occlusion. The thickness of the vessel wall has increased severalfold in the growing collateral vessel compared with the same vessel before occlusion, whereas the lumen has only doubled.
The potential molecular base for translating mechanical force into proliferation and remodeling of collateral vessels also remains to be uncovered. Numerous studies, however, have rendered biochemical pathways of how mechanical forces influence cell shape and function (1, 6, 12, 15, 18–20, 23, 27, 30).

The model presented in this paper will allow us to describe the process of arteriogenesis in greater detail. Bearing in mind that all placebo-controlled clinical trials that were based on the concept of inducing angiogenesis to improve vascularisation of ischemic tissue failed to show any significant or lasting effects, the concept of arteriogenesis might aid in finding feasible therapeutical concepts (8, 28).

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