Overexpression of Csx/Nkx2.5 and GATA-4 Enhances the Efficacy of Mesenchymal Stem Cell Transplantation After Myocardial Infarction

Xi-ren Gao, PhD; Yu-zhen Tan, PhD; Hai-jie Wang, PhD

Background: The high death rate of the transplanted stem cells in the infarcted heart and low efficiency of differentiation toward cardiomyocytes show that mesenchymal stem cell (MSC) transplantation after myocardial infarction (MI) is not effective. Csx/Nkx2.5 and GATA-4 are considered to be key regulators of cardiogenesis. The aim of the present study was to investigate the effect of transplanting MSC overexpressing Csx/Nkx2.5 and GATA-4 (MSCs-CG) after MI.

Methods and Results: According to acridine orange/ethidium bromide staining, MSCs-CG were more resistant to anoxia as compared with MSCs in vitro. In a mouse MI model, ejection fraction and fractional shortening were higher in the MSC-CG group than in the MSC or phosphate-buffered saline group. Wall thickness of the infarct area was increased and collagen deposition was clearly reduced in the MSC-CG group as compared with the other groups. There were more surviving MSCs in the MSC-CG group than in the MSC group. Most of the Y chromosome-positive cells expressed cardiac troponin T and connexin43 (Cx-43). Cx-43 was localized between Y chromosome-positive cells and recipient cardiomyocytes. Microvessel density in the peri-infarct regions and infarct regions increased significantly in the MSC-CG group.

Conclusions: Transplantation of MSCs overexpressing Csx/Nkx2.5 and GATA-4 represents a new treatment strategy with the potential to improve cardiac function after MI. (Circ J 2011; 75: 2683–2691)

Key Words: Cell transplantation; Csx/Nkx2.5; GATA-4; Mesenchymal stem cells; Myocardial infarction

Myocardial infarction (MI) results in a sequence of events that lead to negative left ventricular (LV) remodeling, LV dilation, and eventual heart failure.1 Stem cell transplantation has been extensively investigated as a therapy to regenerate cardiac tissue after MI.2,3 A number of studies have suggested that mesenchymal stem cells (MSCs) could differentiate into cardiomyocytes both in vitro and in vivo.4 One possible advantage of MSCs is their ability to be either autotransplanted or allotransplanted, because some reports have suggested that they may be relatively privileged in terms of immune compatibility.5 Most studies on MSC therapy in experimental animal models and patients with acute MI have shown an improvement in cardiac function,6,7 signifying the safety and feasibility of this approach. The low efficiency of MSC differentiation in adopting the cardiac phenotype, however, affects the effectiveness of transplantation.8 We have found that biomaterials could promote the survival and cardiomyogenic differentiation of transplanted stem cells.9,10 Genetic strategies could help to improve survival and the rate of differentiation.11 Nkx2.5 appears to mark the earliest embryonic heart field and to be capable of initiating the cardiogenic differentiation program. Transplanted cells expressing high levels of Nkx2.5 express cardiac genes even in ectopic locales. Fibroblasts transfected with myc-tagged Nkx2.5 express cardiac genes.12 Myocardin expression is regulated by Nkx2.5 and required for cardiomyogenesis.13 Targeted gene mutation of Nkx2.5 arrests cardiac development at the looping stage.14 GATA-4 is considered to be a key regulator of cardiogenesis. The null mutation of GATA-4 results in abnormal ventral folding of the embryo, failure to form a single ventral heart tube, and lethality by embryonic day (E) 10.5.15 In humans, mutations in GATA-4 result in congenital cardiomyopathies, including valve and septal defects.16,17 The overexpression of GATA-4 enhances the differentiation of P19 cells into cardiac myocytes, whereas its downregulation suppresses this trend.18

Received March 3, 2011; revised manuscript received June 16, 2011; accepted June 30, 2011; released online August 9, 2011. Time for primary review: 19 days
Department of Anatomy, Histology and Embryology, Shanghai Medical School of Fudan University, Shanghai (X.G., Y.T., H.W.); Department of Anatomy, Qiqihar Medical College, Qiqihar (X.G.), China
This work was supported by grants from the Natural Science Foundation of China (No. 30570948).
Mailing address: Hai-jie Wang, PhD, Department of Anatomy, Histology and Embryology, Shanghai Medical School of Fudan University, 138 Yixueyuan Road, Shanghai 200032, China. E-mail: xrgao2008@163.com
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The increased frequency of cardiomyogenic differentiation of 9-15 c cells was successfully achieved in vitro by forced expression of Csx/Nkx2.5 and GATA4.19

In the present study, we tested the hypothesis that MSC grafts overexpressing Csx/Nkx2.5 and GATA-4 may be more resistant to apoptosis and therefore more easily differentiated to cardiomyocytes in the microenvironment after MI, eventually leading to decreased infarct size and improved cardiac function. MSC differentiation was evaluated on fluorescence in situ hybridization and immunohistochemistry staining after MI. The survival of MSCs and neovascularization were also examined.

Methods
All experiments were approved by the Institutional Animal Care and Use Committee of Fudan University. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

MSC Isolation and Culture
Isolation and purification of MSCs from C57B6 mice were performed as described previously.20 In brief, MSCs were collected from aspirates of the femurs and tibias of C57B6 mice with 10 ml of MSC medium consisting of Dulbecco’s modified Eagle's medium (DMEM)-low glucose. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. After 72 h, non-adherent cells were discarded, and the adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). Fresh complete medium was added and replaced every 2 or 3 days for approximately 10 days.

Cloning of Csx/Nkx2.5 and GATA-4 cDNAs
The full open reading frames of mouse Csx/Nkx2.5 and GATA-4 cDNAs were cloned using reverse transcription–polymerase chain reaction (RT-PCR) from poly(A) RNA obtained from the hearts of fetal mice. The primers were as follows: Csx/Nkx2.5: sense, 5'-GGCTCTTTCCCTACCAGGCTCGG-3'; GATA-4: sense, 5'-TGAAACCTGCGTCGCCACCATGT-3'; anti-sense, 5'-TAGTTCTTGTCTGCTGCTGCTCA-3'; anti-sense, 5'-GGCGCTGATTACGCGGTGATTATG-3'. The PCR products were subcloned into pGEM-T vector (Promega, USA). DNA sequencing confirmed that the plasmids contained full-length fragments of the mouse Csx/Nkx2.5 and GATA-4 coding regions.

Retroviral Transduction
Retrovirus expressing Csx/Nkx2.5 and GATA-4 was constructed using a murine stem cell virus (pMSCV) retroviral expression system (Clontech). Virus packaging was carried out by transfecting pMSCV vectors into 293T packaging cells according to the manufacturer’s instructions (Clontech). After 48 h, supernatants were filtered and incubated with MSCs in the presence of 8 μg/ml polybrene (Sigma). Stable Csx/Nkx2.5- and GATA-4-expressing clones were acquired by selection with puromycin (3 μg/ml; Sigma) and verified on quantitative real-time PCR and western blotting.

Quantitative Real-Time PCR
Total RNA from cells was isolated using the Rneasy mini kit (Qiagen). Complementary DNA was synthesized in a 20 μl reaction mixture using SuperScript III First-Strand Synthesis for RT-PCR (Invitrogen). An aliquot of the cDNA was amplified using Taq DNA polymerase (Invitrogen) in the presence of 1 μmol/L sense and anti-sense primers. The primers included the following: Csx/Nkx2.5: sense, 5'-TGGCGTCTGGGAGCTGTCTGTCTG-3'; anti-sense, 5'-GAGCTGTTCTGGCTCCCGCTG-3'; GATA-4: sense, 5'-TACATGCGCAGCTGGGAGCA-3'; anti-sense, 5'-TGGGTTACGCTGGAGGAC-3'. Quantitative real-time PCR was carried out on the iQ5 real-time system with iQ SYBR Supermix (Bio-Rad). Expression of each target mRNA relative to GAPDH was calculated.

Western Blot Analysis
Western blots were performed using whole-cell extracts according to the standard protocol.19 Denatured nuclear protein (60 μg) was separated using sodium dodecysulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Bio-Rad) and probed with antibodies against Nkx2.5 (Santa Cruz) or GATA-4 (Santa Cruz) and then horseradish peroxidase (HRP)-conjugated anti-goat or rabbit IgG. The bands were isolated using the ECL Plus kit (GE Healthcare). Blots were analyzed on densitometry with NIH Image software (AlphaEase FC, version 6.0.0).

Cytoprotective Effects of Csx2.5/Nkx2.5 and GATA-4 Overexpression
To determine the cytoprotective effects of Csx2.5/Nkx2.5 and GATA-4 overexpression for MSCs, the oxygen and glucose deprivation (OGD) model was established in vitro. After culturing for 24 h in 96-well culture plates, the cell culture media from both groups of MSCs and MSCs overexpressing Csx/Nkx2.5 and GATA-4 (MSCs-CG) were replaced with glucose- and serum-free DMEM (Gibco, USA). The plates were then placed in a 37°C anoxia chamber saturated with 95% N2/5% CO2. At 12 h after incubation, the cells from each group were stained with acridine orange/ethidium bromide (AO/EB). Both AO and EB were dissolved in 0.01 mol/L PBS at a concentration of 100 μg/ml. The fluorescent dyes AO and EB were added to the supernatant. Digital images were acquired, and the number of differentially stained cells in random fields of 500 cells was determined. The data are expressed as the percentages of live, apoptotic and necrotic cells.

MI Model and Cell Transplantation
MI was created in 40 female 10-week-old C57B6 mice by ligation of the left coronary artery.21 Thirty minutes later, the mice were randomly divided into PBS, MSC and MSC-CG groups. In the MSC or MSC-CG group, a total of 5×106 MSCs or MSCs-CG were suspended in 80 μl of PBS and injected into the border of the infarcted myocardium through a 30-G needle while the heart was beating. PBS (80 μl) was injected into animals that served as the control group. The injection was done at 4 sites, with 20 μl at each site.

Echocardiography
To evaluate the recovery of cardiac function, transthoracic echocardiography was performed at 4 weeks after transplantation. After adequate 2-D images were obtained, the M-mode cursor was positioned to the parasternal long axis view at the papillary muscle level. LV internal dimensions as well as LV end-systolic (LVED) and end-diastolic (LVEDD) diameters were measured from at least 3 consecutive cardiac cycles. Ejection fraction (EF) and fractional shortening (FS) were calculated as measures of systolic function using the equations EF= [(LVEDD−LVED)/LVEDD]×100 and FS= (LVEDD−LVED)/LVEDD×100, respectively. The EF and FS results are expressed as percentages.
Histology
To detect fibrosis in cardiac muscle, the hearts were excised, cut transversely, embedded in paraffin and stained with Masson’s trichrome. The blue area was regarded as fibrotic tissue. Five different ventricular slices covering the entire infarcted area from the apex to the site of occlusion were scanned and computerized with a digital image analyzer (ImagePro plus). The collagen volume fraction was calculated as the sum of all areas containing connective tissue divided by the total area of the image. Wall thickness of the infarct area was also measured using the image analyzer.

Survival of Transplanted Cells
PCR amplification was used to identify surviving transplanted male MSCs through the presence of a Y chromosome. LV tissue from the injection site was isolated and refrigerated in liquid nitrogen. Genomic DNA was extracted for the PCR template. Genomic DNA templates (100 ng) from different groups were used in PCR reactions (50 μl) with mouse SRY primers according to the protocols published by Müller-Ehmsen et al.\(^\text{22}\) The intensities of the ethidium bromide-stained SRY bands were quantified using NIH Image (Wayne Rasband, NIH, USA) and subsequently normalized to β-actin mRNA levels.

Fluorescence In Situ Hybridization and Immunohistochemistry
To detect the transplanted cells, in situ hybridization was performed using a mouse Y chromosome-specific gene. Frozen sections were fixed with methanol and acetic acid (3:1) for 30 min at 4°C, and the slides were then immersed in denaturation buffer (70% formamide) at 95°C for 5 min to denature the fixed chromosome specimens. After denaturation, the samples were dehydrated through a gradient ethanol series of 70%, 80%, 95% and 100% at –20°C (for 5 min each) and then air-dried. The biotin-labeled Y chromosome-specific probe (Roche, Germany) was denatured at 95°C for 5 min. The sections were incubated with the denatured probe at 42°C overnight in a moist chamber. After washing twice with 2× saline sodium citrate buffer, the sections were incubated with streptavidin-FITC (BioLegend, USA) for 30 min at 37°C in the dark. To determine the differentiation of transplanted cells into cardiomyocytes and the relationship between differentiated cells and host cardiomyocytes, immunohistochemistry for cardiac troponin T (cTnT) and connexin43 (Cx-43) was performed. The same sections were treated with blocking solution for 30 min at 37°C and then incubated with human anti-cTnT antibody (1:300, Santa Cruz) or rabbit anti-Cx-43 antibody (1:200, Abcam, USA) at 4°C overnight. The sections were incubated with donkey anti-human or goat anti-rabbit Cy3-conjugated.
antibody (Jackson, USA) for 30 min at 37°C. After washing, the nuclei were counterstained with DAPI (Molecular Probes, USA). The presence of the Y chromosome and cTnT or Cx-43 in the cells was determined under a fluorescence microscope.

Density of Microvessels
For immunohistochemical analysis of microvessel density, the tissue sections were prepared and stained with CD31 (BD Biosciences). After treatment with hydrogen peroxide to block endogenous peroxidase activity, the sections were incubated...
with rabbit CD31 at 4°C overnight, then incubated with goat anti-rabbit HRP-labeled secondary antibody and treated with 3,3-diaminobenzidine. Finally, the nuclei were counterstained with hematoxylin. At least 2 microscopic fields in the peri-infarct and infarct regions were randomly selected and counted in 3 different sections from each animal. The results were expressed as the number of microvessels per 400× field.

Statistical Analysis
The data are expressed as mean±SD. To analyze the data statistically, we used Student’s t-test and 1-way analysis of variance (ANOVA) with Scheffe’s post-hoc multiple-comparison analysis. P<0.05 was considered to be statistically significant.

Results
Characterization of MSCs-CG
Retroviral-mediated transduction and expression of Csx/Nkx2.5 and GATA-4 were confirmed on real-time PCR and western blotting. Quantitative real-time PCR data indicated that expression of Csx/Nkx2.5 was 259-fold higher and expression of GATA-4 was 238-fold higher in MSCs-CG (Figure 1A). MSCs-CG also exhibited higher levels of Csx/Nkx2.5 and GATA-4 protein (Figure 1B).
Figure 6. Survival of transplanted cells in vivo. (A) RT-PCR for the SRY gene using left ventricular tissue from each group. (B) Quantitative analysis of SRY gene expression. MSC, mesenchymal stem cell; MSC-CG, MSCs overexpressing Csx/Nkx2.5 and GATA-4; PBS, phosphate-buffered saline. *P<0.01 vs. the MSC group. n=10.

Figure 7. Cardiomyogenic differentiation of donor cells. (A) Expression of cTnT by Y chromosome-positive cells. Most Y chromosome-positive cells (green, arrows) in the infarct and border zones co-expressed cTnT. Scale bar, 20 μm. (B) Expression of Cx-43 by Y chromosome-positive cells. Most of the transplanted cells (green, arrows) expressed Cx-43 (red, arrowheads). Cx-43 was located between Y chromosome-positive cells and recipient cardiomyocytes. Scale bar, 20 μm. MSC, mesenchymal stem cell; MSC-CG, MSCs overexpressing Csx/Nkx2.5 and GATA-4.
Csx/Nkx2.5-GATA-4 Enhance Transplantation Efficacy

To investigate the resistance of MSCs-CG to oxidative stress, MSCs and MSCs-CG were exposed to anoxia for 12 h and stained with AO/EB. MSCs displayed morphological changes of apoptosis and necrosis after treatment with OGD. Overexpression of Csx/Nkx2.5 and GATA-4, however, partially prevented this phenomenon. The number of surviving cells was greater in the MSC-CG group (70.82±7.90%) than in the MSC group (46.76±7.89%, P<0.05). The numbers of apoptotic cells (14.99±2.52%) and necrotic cells (14.86±5.80%) decreased significantly in the MSC-CG group as compared with the MSC group (23.45±6.47%, P<0.05; 29.80±4.89%, P<0.05; Figure 2).

Improvement of LV Contractile Function
At 4 weeks after transplantation, EF and FS had improved more in the MSC and MSC-CG groups than in the PBS group. On echocardiography EF and FS were higher in the MSC-CG group (68.37±2.71, 32.31±23.55) than in the MSC group (56.38±3.32, 23.55±3.33; P<0.01; Figure 3).

Myocardial Fibrosis and Wall Thickness of the Infarct Segment
Masson’s trichrome staining showed extensive myocardial fibrosis in the PBS group. There was more regenerated myocardium, however, in the infarction area in the MSC and MSC-CG groups at 4 weeks after transplantation. Transplantation of MSCs overexpressing Csx/Nkx2.5 and GATA-4 significantly attenuated the development of myocardial fibrosis (Figure 4A). Quantitatively, the collagen volume fraction in the MSC and MSC-CG groups was significantly smaller than in the PBS group. Compared with the MSC group, the collagen content of the MSC-CG group was much lower (Figure 4B). The thickness of the old infarct wall was greater in MSC-CG-treated mice than in the MSC-treated or the PBS-treated group, although MSC treatment increased wall thickness of the infarct segment (Figure 5).

Survival of Transplanted Cells, as Determined on PCR
LV tissue from 10 mice in each group was used for detecting the SRY gene on PCR. All tissues from animal groups receiving MSCs or MSC-CG were positive, confirming the presence and survival of the transplanted cells, while all tissues from the PBS animals that did not receive graft cells were negative (Figure 6A). Quantitative analysis showed that there were more surviving transplanted cells in the MSC-CG group than in the MSC group (Figure 6B).

Cardiomyogenic Differentiation of Donor Cells In Vivo
Y chromosome fluorescence in situ hybridization demonstrated that there were more Y chromosome-positive cells in the MSC-CG group than in the MSC group at 4 weeks after transplantation, and most of the cells expressed cTnT and Cx-43. Cx-43 is located between Y chromosome-positive cells and recipient cardiomyocytes. The differentiated cells were aligned in parallel and connected with host cardiomyocytes (Figure 7).
Angiogenesis

To detect endothelial cells, the angiogenic effect was determined on immunostaining specific for CD31 expression at 4 weeks after transplantation (Figure 8A). There was a significant increase in microvessel density in the MSC group (69.8±12.9, P<0.01) and in the MSC-CG group (113.4±18.4, P<0.01) as compared with the PBS group (39.3±13.9). The microvessel density in the MSC-CG group was higher than in the MSC group (Figure 8B).

Discussion

The high death rate of the transplanted stem cells in the infarcted heart and the low efficiency of differentiation toward cardiomyocytes influence the outcome of stem cell transplantation for treatment of MI.23 More than 90% of autologous skeletal myoblasts underwent apoptosis or necrosis at 24 h after transplantation.24 Therefore, a strategy to improve the survival and differentiation of transplanted cells in the infarction site is critical for improving the efficiency of stem cell therapy.

Overexpression of GATA-4 enhances the differentiation of P19 cells into cardiac myocytes and suppresses the differentiation of P19 cells into the cells generated from the endoderm.25 Nkx2.5 is one of the early indicators of embryonic cardiac development.26 The transfection of Nkx2.5 into zebrafish fibroblast cells can induce the expression of markers of cardiac myocyte differentiation; this suggests that non-cardiac cells overexpressing Nkx2.5 can also be used to observe the process of heart differentiation.26 We demonstrated that transplantation ofMSCs overexpressing Csx/Nkx2.5 and GATA-4 decreased collagen deposition and improved heart function more efficiently compared with the transplantation ofMSCs and PBS. Csx/Nkx2.5 and GATA-4 overexpression enhanced the survival and differentiation ofMSCs and played a key role in improving the efficiency of stem cell transplantation. The present in vitro results indicate that some empty vector-transfected MSCs underwent apoptosis and necrosis (data not shown), which is consistent with previous research. As demonstrated by Jiang et al, compared with non-transfected normal cells, the number of surviving cells in the group of empty vector-transfected MSCs was significantly reduced.27 Therefore, we used the cells without transfection as the control group. Nkx2.5 and GATA-4 are considered to be key regulators of cardiogenesis. Transplantation ofMSCs overexpressing Csx/Nkx2.5 or GATA-4 alone may also enhance the survival and differentiation ofgraftedMSCs. Transplantation ofMSCs co-transfected with Csx/Nkx2.5 and GATA-4, however, may be more effective for cardiac repair and function recovery. The increased frequency of cardiomyogenic differentiation of9-15c cells was successfully achieved in vitro by forced expression ofCsx/Nkx2.5 and GATA4.19 Further study needs to be done to investigate the individual effect of Csx/Nkx2.5 or GATA-4 overexpression.

Recent evidence has suggested that GATA-4 is also one of the anti-apoptotic factors regulating cardiac myocyte survival.28,29 Restoration of GATA-4 activity attenuated the apoptosis ofcardiac myocytes induced by anthracyclines.29 In the present study, Csx/Nkx2.5 and GATA-4 overexpression promoted MSC survival in vitro and in the hostile environment after MI. After treatment with OGD, the cells displayed obvious chromatin condensation and cell nuclear fragmentation. The MSC-CG group, however, resisted apoptosis and necrosis induced by OGD treatment. On PCR to identify the Y chromosome, the survival of donor MSCs was higher in the MSC-CG group. Cytoprotective effects ofCsx/Nkx2.5 and GATA-4 overexpression contribute to the survival and differentiation ofMSCs.

The therapeutic potential ofMSC-CG transplantation has also been attributed to increased angiogenesis. GATA-4 increases MSC survival and paracrine activity, which promotes neovascularization in the ischemic border zone and infarct area, thereby enhancing cardiac functional recovery.30 An increase in blood flow to the infarct is thought to salvage at-risk cardiomyocytes and produce a smaller infarct. Angiogenesis could also enhance the survival of donor MSCs. We performed a comprehensive evaluation of the distribution profile ofangiogenesis in the recipient heart. Many more newly formed vessels were observed in the peri-infarction and infarction regions in the MSC-CG group. There was an obvious propensity ofCD31-positive cells in MSC-CG group heart tissue sections as compared with those obtained from animals in the MSC and PBS groups.

Although angiogenesis and paracrine action can improve cardiac function through indirect effects, they can only save the dying cardiomyocytes or mobilize cardiac stem cells within the recipient heart. The number of cardiac stem cells produced by mobilization is few, and the damaged cardiomyocytes cannot be replaced effectively. The differentiation of transplanted stem cells into cardiomyocytes in the infarcted zone to replace the damaged recipient cardiomyocytes and improve heart function is the most important objective ofstem cell transplantation. Quevedo et al reported that allogeneicMSCs can reduce the infarcted area and improve cardiac function in chronic ischemic cardiomyopathy via their trilineage differentiation capacity, including cardiomyogenesis.31 The rate ofcardiomyogenic differentiation, however, was difficult to discern. In the present study, at 4 weeks after transplantation, there were more donor cells expressing αTnT and Cx-43 in the MSC-CG group than in the MSC group. The engrafted cells underwent cardiomyogenic differentiation, formed mature muscle fibers and formed gap junctions with the myocardium of the recipient. Csx/Nkx2.5 and GATA-4 are 2 cardiac-enriched transcription factors that are expressed in precardiac mesoderm from the very early developmental stage.32,33 Both Csx/Nkx2.5 and GATA-4 are required for the cardiac differentiation ofP19CL6 cells derived from embryonic teratocarcinoma cells.34 Therefore, overexpression ofCsx/Nkx2.5 and GATA4 efficiently promotes the differentiation ofMSCs into cardiomyocytes in the microenvironment after MI. Further studies are necessary, however, to elucidate the contractile nature of the newly formed muscle fibers after connection with the myocardium of the recipient.

In conclusion, overexpression ofCsx/Nkx2.5 and GATA-4 enhanced the survival and differentiation ofMSCs. Transplantation ofMSC-CG was more conducive to ameliorate LV remodeling and improve LV function by promoting the survival and cardiomyogenic differentiation ofMSCs and inducing angiogenesis. These observations suggest thatMSCs genetically modified to overexpress Csx/Nkx2.5 and GATA-4 could significantly advance the efficacy of stem cell therapy.

Acknowledgments

This work was supported by grants from the Natural Science Foundation of China (No. 30570948).

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