Lead acetate reduces the ability of human umbilical cord mesenchymal stem cells to support hematopoiesis *in vitro*

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Received March 5, 2012; Accepted July 23, 2012

DOI: 10.3892/mmr.2012.1014

Abstract. Plumbum (Pb) is a heavy metal toxin that causes many pathophysiological effects in various systems of the human body. It has previously been reported that excessive lead trioxide causes hematopoietic system toxicity. Mesenchymal stem cells (MSCs), as cells with self-renewal and multipotent differentiation potential, play a supportive role in hematopoietic function. Lead is well known to interfere with hemoglobin synthesis and affect erythrocyte morphology and survival. MSCs and the cytokines secreted by MSCs are the important components of the hematopoietic microenvironment. Thus, we hypothesized that lead may cause damage to MSCs, which may provide a new understanding of the mechanism of lead toxicity in the hematopoietic system. In the present study, cell count, MTT assay, apoptosis assay, osteogenic differentiation, cell histochemical staining, cell cycle analysis, colony forming assay and RT-PCR were used. The results showed that the proliferation of umbilical cord MSCs (UCMSCs) was affected if the concentrations of lead were higher than 10 μM. Following osteogenic differentiation, the rate of alkaline phosphatase and Von Kossa stain positivity in the experimental group was lower than that in the control group. In conclusion, these results demonstrate that lead suppresses the self-renewal and multipotent differentiation potential of UCMSCs, and induces an adverse effect on the expression of UCMSCs cytokines. MSCs also have a hematopoiesis-promoting function that is capable of supporting colony formation of bone marrow cells. Furthermore, we found that following treatment with lead acetate, the supportive function of UCMSCs on colony formation was inhibited. Taken together, lead acetate has a toxic effect on the self-renewal, multipotent differentiation potential and hematopoiesis-promoting function of UCMSCs.

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Key words: human umbilical cord mesenchymal stem cells, lead acetate, hematopoiesis-supportive function

Introduction

Plumbum (Pb), one of the most widespread pollutants in the world, induces oxidative stress and dysfunction in many cell types (1). For humans, the major sources of exposure to lead compounds include air, dust, food, water and batteries (2-5). Lead has been found to cause neurotoxicity, reproductive toxicity, liver toxicity, kidney damage, hematological dysfunctions and immunotoxicity (3). Among all of these, the hematological system has been proposed as an important target for lead-induced toxicity. Approximately 99% of the lead present in the blood is bound to erythrocytes. Moreover, erythrocytes may spread lead to different organs of the body. The effect of lead on health depends not only on its total dose and the length of exposure but also on the physical and chemical state of the element and the physiological status and age of the individual (6,7). The most vulnerable groups at risk of harm from lead exposure are fetuses and children of preschool age (6,8). A previous report demonstrated that iron deficiency was associated with higher blood lead levels in children (9).

In the current study, we used inorganic lead and lead acetate to investigate the effects of lead on the proliferation, osteogenic differentiation and hematopoiesis-supportive function of mesenchymal stem cells (MSCs). Stem cells may provide a model of cells undergoing differentiation and proliferation for toxicology assays (10). For example, embryonic stem cells have already provided new assays to predict embryo-fetal developmental toxicity *in vitro* (11). However, certain problems are encountered with the application of embryonic stem cells, including complicated culture methods, high costs and ethical concerns. MSCs, a type of adult stem cell, are capable of self-renewal, have multipotent differentiation potential (12,13), may be isolated from different tissue sources, and are easily isolated from bone marrow (BM) and expanded *in vitro* (14-17). They are capable of supporting hematopoiesis (18), enhancing angiogenesis (19) and repairing tissue (20). Thus, it is clear that any cellular stress in MSCs is capable of markedly diminishing the hematopoietic system. The hematopoietic system is vulnerable to environmental toxins and heavy metals such as cadmium (21) and lead (22). They interfere in the hematopoietic system through the damage of genetic material of stromal and hematopoietic cells and the alteration of

cytokine homeostasis. It is, therefore, necessary to explore the lead-induced response of MSCs.

Materials and methods

Cell culture. Human umbilical cord mesenchymal stem cells (UCMSCs) were isolated from umbilical cord Wharton's jelly. Umbilical cords were obtained from the Fourth Hospital of Zhenjiang, China. Umbilical cords were rinsed twice by phosphate-buffered saline (PBS) in penicillin and streptomycin. The tissues were minced into 1-2 mm^3 fragments. They were cultured in a 37˚C incubator in plates with low-glucose Dulbecco's modified Eagle's medium (L-DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin. The medium was changed every 3 days. After approximately two weeks, there were cells at the edge of the tissue fragments. When UCMSCs grew to 70% confluence, they were detached by 0.25% Trypsin-EDTA and reseeded in a flask of 5,000 cells/cm^2 for optimal proliferation. The experimental protocol was approved by the Jiangsu University ethics committee and informed consent was obtained.

Cell count. UCMSCs were seeded at $1x10^4$ cells/cm^2 in 24-well plates with L-DMEM containing 10% FBS. After one day for attachment and growth, the medium was replaced with fresh L-DMEM supplemented with 10% FBS containing 0, 20, 40, 60, 80 and 100 μM lead acetate for 24, 48 and 72 h. Cells were harvested and counted by quantitative cytometry.

Quantifying cell viability by MTT assay. UCMSCs were grown in 96-well culture plates. After one day for attachment and growth, cells grown in 96-well plates with 200 μl L-DMDM were exposed to various concentrations of lead acetate (0, 20, 40, 60, 80 and 100 μM) for 72 h. MTT (20 μl) was added to each well for the final 4 h. When the reaction was terminated, all the solution was discarded and 150 μl DMSO was added to each well. The 96-well plate was agitated to ensure that complete solubilization of the purple formazan crystals occurred. Absorbance at 490 nm was measured using an ELISA reader.

Cell cycle assay. UCMSCs were treated with lead acetate for 24, 48 and 72 h. Cells were harvested and washed twice with PBS and stained with propidium iodide (PI) (Sigma-Aldrich, USA) for 30 min in dark conditions. The stained cells were analyzed by flow cytometry.

Apoptosis assay. UCMSCs were treated with lead acetate for 48 h. Following treatment, the cells were trypsinized with 0.25% trypsin-EDTA, washed twice with PBS and stained with PI and annexin-V-FITC according to the manufacturer's instructions. The stained cells were analyzed by flow cytometry.

Osteogenic differentiation. UCMSCs were seeded at a density of $3x10^4$ cells/cm^2 and stimulated with osteogenic induction medium for 14 days. The osteogenic medium consisted of low-glucose DMEM, 10% FBS, 100 nM dexamethasone, 10 mM sodium β-glycerophosphate and 0.05 mM-ascorbic acid 2-phosphate (all from Sigma), and was replaced every 3 days.

Cell histochemical staining. In the differentiated UCMSCs, osteogenic characteristics were confirmed by alkaline phosphatase (ALP) expression using histochemical staining and the observation of the hydroxyapatite matrix using Von Kossa staining. Cells were fixed with fixing agent and stained with ALP according to the manufacturer's instructions. The Von Kossa stain was performed according to the protocol described previously (23,24) with a few modifications. The cells were fixed with 4% paraformaldehyde for 5 min at room temperature, stained for 20-60 min with 2% silver nitrate (Sigma-Aldrich) and then treated with 5% sodium thiosulfate (Sigma-Aldrich) for 2 min.

RT-PCR. Total RNA was extracted from UCMSCs and UCMSCs treated with lead acetate using TRIzol reagent (Invitrogen, USA). cDNA was obtained from 1 μg total RNA using an oligo-dT primer in a reaction volume of 20 μl, using a reverse transcription kit according to the manufacturer's instructions (Fermentas). The cDNA samples were subjected to polymerase chain reaction (PCR) using specific primers. The conditions of PCR were as follows: Initial denaturation at 94˚C for 5 min, denaturation at 94˚C for 30 sec, annealing at 55-70˚C for 30 sec (see Table I for temperatures used), extension for 30 sec at 72˚C for 30 cycles, and a final polymerization at 72˚C for 10 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide staining and photographed under UV transillumination. β-actin was used as a control. The specific primers were designed as shown in Table I.

Colony formation assay. UCMSCs were seeded in a 6-well plate with L-DMEM medium supplemented with 10% FBS. After one day for attachment and growth, the medium was replaced with fresh L-DMEM supplemented with 10% FBS containing 60 μM lead acetate for 72 h. There was no lead acetate in the control group. Following treatment, the medium was removed and cells were washed with PBS 2-3 times. Sterile 3% agar was prepared and incubated at 65˚C in a waterbath. Bone marrow cells were added to a 10 ml tube with 9 ml L-DMEM medium supplemented with 10% FBS and incubated at 37˚C. In total, 1 ml 3% agar was added to 9 ml cell suspension and mixed gently by swirling, and 1 ml was quickly added to each of the wells of the prepared 6-well plate. Plates were incubated at 37˚C for 14 days. Colonies were counted using a microscope.

Results

Effect of lead acetate on cell proliferation. UCMSCs were exposed to various concentrations of lead acetate (0, 20, 40, 60, 80 and 100 μM) for 24, 48 and 72 h, and counted with a cytometer. The number of cells was significantly decreased in a dose-dependent manner following lead acetate treatment for 24, 48 and 72 h (Fig. 1A). To further investigate the cytotoxic effects of lead acetate on cell proliferation, we used the MTT assay to test lead acetate-induced toxicity in UCMSCs. After 72 h of lead acetate treatment, cell viability was markedly decreased in a dose-dependent manner (Fig. 1B).

Effect of lead acetate-induced apoptosis in human UMSCs. Treatment of lead acetate at the concentration of 60 μM showed an increase in annexin V-positive cells over the untreated

Table I. Specific primers for control and target genes.

ID	Gene	Primers sequence (5' to 3')	Size (bp)	Annealing temp (˚C)
1	Ang-1-F Ang-1-R	AGAGGCACGGAAGGAGTGTG CTATCTCCAGCATGGTAGCCG	249	57
2	TPO-F TPO-R	ATTGCTCCTCGTGGTCAT CTCCTCCATCTGGGTTTT	220	56
3	Flt3-ligand-F Flt3-ligand-R	CTGGAGCCCAACAACCTATC TCTGGACGAAGCGAAGACA	353	60
4	SCF-F SCF-R	TGGATAAGCGAGATGGTA TTCTGGGCTCTTGAATGA	189	54
5	Ang-2-F Ang-2-R	GGATCTGGGGAGAGAGGAAC CTCTGCACCGAGTCATCGTA	371	55
6	VEGF-F VEGF-R	CCTTGCTCTACCTCCAC ATCTGCATCCTGTTGGA	280	61
7	ALP-F ALP-R	AGCTTCAAACCGAGATACAA ATTCTGCCTCCTTCCACC	220	56.5
8	β-actin-F β-actin-R	CACGAAACTACCTTCAACTC CATACTCCTGCTTGCTGATC	256	56

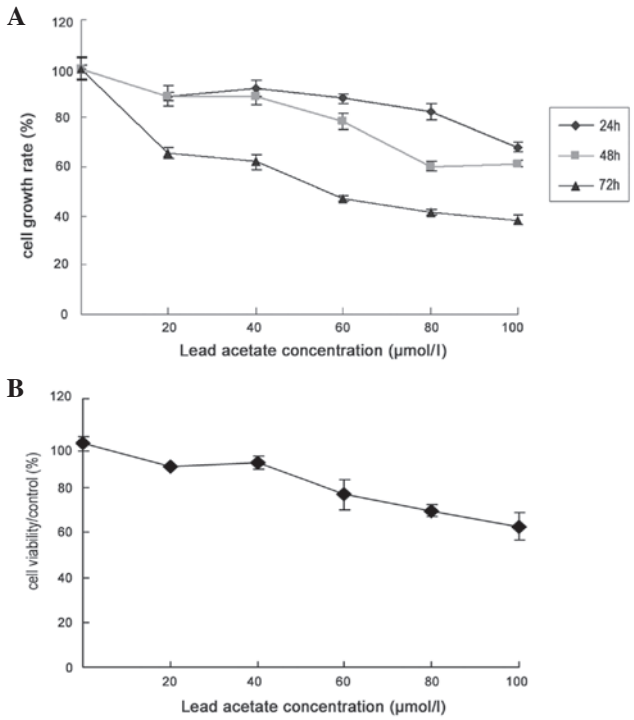

Figure 1. Effect of lead acetate on the growth rate of MSCs. (A) Cell growth rate was assessed by cell count. The cells were treated by lead acetate (20, 40, 60, 80 and 100 μM) for 24, 48 and 72 h. The number of untreated cells were as 100% and the bars represent the means ± SE. (B) Cells were treated with lead acetate (20, 40, 60, 80 and 100 μM) for 72 h, and cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide) assay. MSCs, mesenchymal stem cells.

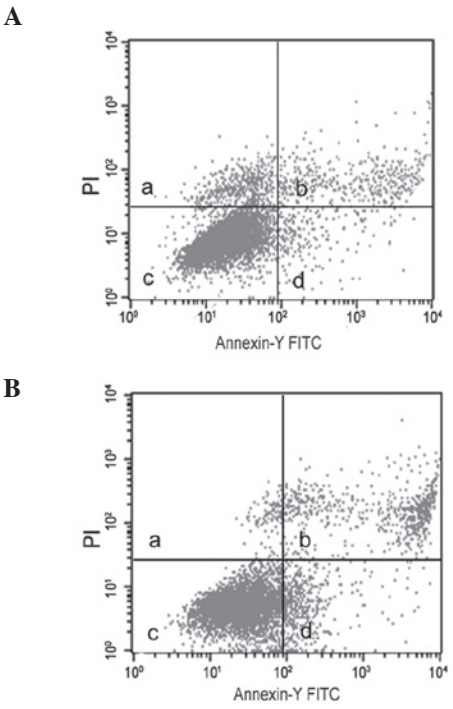

Figure 2. Effects of lead acetate-induced apoptosis in human UMSCs. Representative scattergrams from the flow cytometry profile represents annexin V-FITC staining in the X-axis and PI in the Y-axis. UMSCs, umbilical cord mesenchymal stem cells; PI, propidium iodide. (A) Control group. (B) Treatment group. The proportion of non-apoptotic cells (c, annexin V-FITC-/PI-), early apoptotic cells (d, annexin V-FITC+/PI-), late apoptotic/necrotic cells (b, annexin V-FITC+/PI+) and dead cells (a, annexin V-FITC-/PI+).

UCMSCs (Fig. 2), which suggested that lead acetate is capable of inducing apoptosis in UCMSCs.

Effect of lead acetate on osteogenic differentiation. In order to investigate the effect of lead acetate on the differentiation potential, UCMSCs were induced to osteogenic differentiation. Following osteogenic differentiation of UCMSCs, the cells were stained with ALP and Von Kossa. Compared with the control, the rates of ALP-positive staining and extracellular matrix (ECM) calcium deposits were reduced following lead

Table II. Effect of lead acetate on supportive function of MSCs on colony formation ability.

Days	Control group	Lead acetate group (60 μM)
7	214±9.5	139±10.7[a]
10	340±15.6	233±3.7[a]
14	393±6.6	272±9.3[a]

Colonies of control and lead acetate (60 μM) groups were expressed as the means ± SE (n=3 for each group). [a]P<0.05 as compared with vehicle control group.

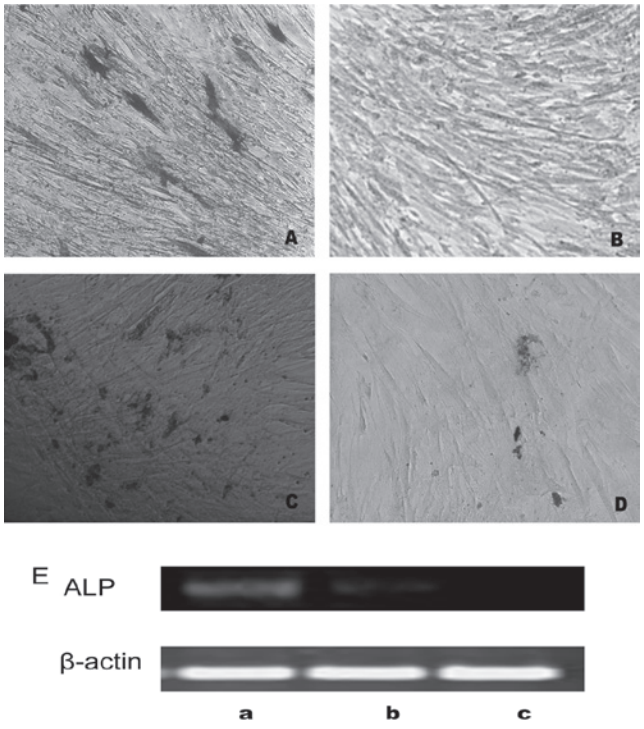

Figure 3. Effects of lead acetate on osteogenic differentiation. (A-B) The cells were stained by alkaline phosphatase. (A) Control; (B) treated group. (C and D) The cells were stained by Von Kossa. (C) Control; (D) treated group. (E) The expression of ALP was determind by RT-PCR following osteogenic differentiation. Lane a, control group; lane b, treated group; lane c, negative control group. ALP, alkaline phosphatase.

acetate treatment (Fig. 3). The gene expression of ALP also revealed a marked downregulation in the experimental group (Fig. 3E). These results indicated that lead acetate inhibited the osteogenic differentiation of MSCs.

Effect of lead acetate on certain genes of hematogenesis. To investigate the effect of lead acetate on the hematopoietic system, the mRNA expression of cytokines was detected in UCMSCs. These cytokines are an important component of the hematopoietic microenvironment as well as MSCs. UCMSCs were treated with lead acetate (60 μM) and RT-PCR was performed to analyze mRNA expression. Following lead treatment, certain genes which were connected with the hematopoietic system and angiopoiesis, including Ang-1,

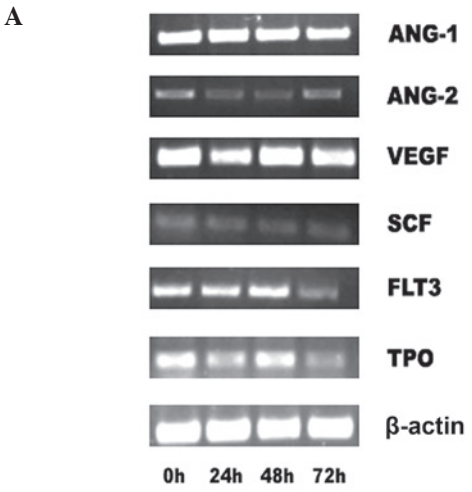

Figure 4. Cytokine mRNA expression in lead acetate-treated MSCs. (A) Cells were treated by 60 μM lead acetate for 24, 48 and 72 h. The mRNA expression of Ang-1, Ang-2, VEGF, SCF, Flt3-ligand, TPO and VEGF were determined by PCR. (B) The fold of gene expression. MSCs, mesenchymal stem cells.

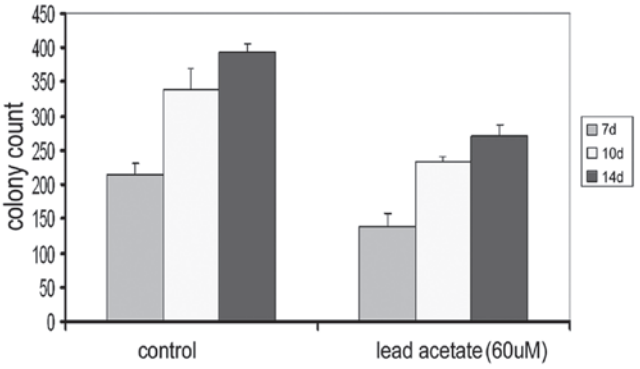

Figure 5. The number of colonies was significantly lower in the lead acetate group than the control group.

Ang-2, VEGF, SCF, Flt3-ligand and TPO, were significantly decreased (Fig. 4). These results indicated that lead acetate could induce hematopoietic system damage through inhibiting the hematopoiesis-supportive function of MSCs.

Effect of lead acetate on the hematopoiesis-supportive function of UCMSCs. MSCs have a hematopoiesis-supportive function *in vitro*. To further prove the reverse effect of lead

acetate on the hematopoiesis-supportive function of MSCs, we detected the supportive function of UCMSCs on the colony formation ability of bone marrow cells following treatment of lead acetate. After 7, 10 and 14 days, we counted the number of colonies in each well and found that the number of cells in the experimental groups were all significantly lower than that of the control group (Fig. 5 and Table II). Following treatment with lead, the supportive function of MSCs on colony formation was inhibited.

Discussion

In previous reports (25), lead concentrations in the bone greatly exceeded the concentrations in soft tissues and were highest in the dense bones. Over 90% of the total body burden of lead in adults was in the bone, of which over 70% was in dense bone (26). Therefore, bone marrow is an important target organ of lead toxicity. Haleagrahara *et al* found that treatment with 500 ppm lead acetate in the drinking water of Sprague-Dawley rats for 14 days showed morphological changes in the bone marrow, and there was very little hematopoietic tissue and a relatively increased amount of fat cells (27). The heavy metal lead is capable of inducing genotoxicity. Following the treatment with lead, there were significant increases in the chromosomal aberration of rat bone marrow cells (28). The ability of the stromal cell layer of bone marrow to display myeloid progenitor cells *in vitro* was reduced when mice were treated with lead (29). The cells and microenvironment of the bone marrow were impaired when exposed to lead. Bone marrow is the site where hematopoiesis occurs. Therefore, lead-induced damage of the hematopoietic system may be associated with the bone marrow. Lead is known to alter the functions of the hematopoietic system. Lead toxicity induces changes in the composition of red blood cell membrane proteins and lipids that inhibit hemoglobin synthesis, limit erythrocyte production and reduce red cell survival (30,31). Furthermore, lead interferes with iron utilization for heme formation in the mitochondria, and radio-iron studies showed that lead competes with iron for incorporation into red blood cells (32).

Bone marrow MSCs are thought to give rise to cells that constitute the hematopoietic microenvironment. MSCs may produce a number of cytokines and ECM proteins and express cell adhesion molecules, all of which are involved in the regulation of hematopoiesis (33). Human MSCs, when injected into the bone marrow cavity of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, differentiate into the various cellular components of the bone marrow stroma, which constitute the functional components of the hematopoietic microenvironment (12).

In our study, lead acetate not only damaged the balance of cell proliferation and induced cell apoptosis, but also affected the potential of differentiation. Following osteogenic induction, we found that the positive rate of ALP in the treatment group was markedly lower than that in the control group. The underlying mechanisms may be complicated and worth further investigation.

MSCs are one of the essential components of the hematopoietic microenvironment. MSCs are capable of secreting certain cytokines associated with hematogenesis. In the present study, we found that the expression of certain UCMSC cytokines was significantly decreased following lead acetate treatment. The cytokine spectrum of MSCs could partly explain the hematopoiesis-supportive function. These results suggested that one mechanism underlying the toxic effects of lead on the hematopoietic system may change the hematopoietic microenvironment.

To further demonstrate this hypothesis, we detected the hematopoiesis-supportive function of MSCs through a colony formation assay *in vitro*, and found that after the treatment of lead acetate the supportive function of MSCs on colony formation was inhibited. The result indicated that lead acetate injured the hematopoiesis-supportive function of UCMSCs. Therefore, the hematopoiesis-supportive function of MSCs was destroyed, which is one of the reasons for the anemia that lead poisoning may produce.

Based on all of the above, we concluded that lead toxicity induces changes in the red blood cell membrane, inhibits hemoglobin synthesis, and then leads to decreased erythrocyte production or reduced red blood cell survival. However, lead interferes with iron utilization for heme formation and competes with iron for incorporation into red blood cells. Furthermore, lead destroys the hematopoiesis-supportive function of MSCs, and all of the above are causes of the anemia that occurs in lead toxicity.

In conclusion, lead acetate could inhibit the proliferation, osteogenic differentiation and hematopoiesis-supportive function of UCMSCs.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant no. 30840053), the Natural Science Foundation of Jiangsu Province (Grant no. BK2008232) and Foundation of the Jiangsu University for senior talent (Grant no. 11JDG0089).

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