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Effect of UA on the Expression of BMP-2 during the Osteogenic Differentiation of Human Bone Marrow Mesenchymal Stem Cells*

XIE Ting-ting[1], YANG Nai-long[1△], XU Li-li[1], WANG Shu-fang[2]

(1 Deparmtment of Endocrinology, Affiliated Hospital of Qingdao University, Qingdao, Shandong, 266000, China;

2 Deparmtment of Endocrinology, Caoxian People's Hospital, Heze, Shandong, 274000, China)

ABSTRACT Objective: To observe effect of uric acid (UA) on expression of bone morphogenetic protein-2 (BMP-2) in differentiation process from human bone marrow mesenchymal stem cells (hBMSCs) to osteoblasts in vitro. **Methods:** hBMSCs were isolated and cultured using the whole bone marrow adherence method. Passage 3 hBMSCs were divided into 5 groups: blank control group (adding complete medium) and osteogenic induction group (adding osteoblast inducing media and 0 mmol/L, 0.2 mmol/L, 0.4 mmol/L, 0.8 mmol/L UA in complete medium). After 14 days of induction, cell morphology was observed under an inverted microscope. Osteogenic ability was identified by alkaline phosphatase (ALP) activity assay and alizarin red stain. BMP-2 mRNA expression was detected by reverse transcription PCR (RT-PCR). **Results:** Passage 3 cells were single long-spindle in shape and gradually formed a whirlpool-shaped arrangement. After induction, the majority cells were from long-spindle to irregular cube in shape, and gradually became paving stone or formed nodules. Among all the groups, the cells in 0.8 mmol/L UA induction medium formed the most nodules. After 14 days of induction, calcium nodules in the blank control group were negtive, however, calcium nodules were dyed orange in osteogenic induction group. The result showed hBMSCs were induced into osteoblasts successfully. ALP activity enhanced gradually with the increase of intervention time and UA concentration ($P<0.05$). RT-PCR results showed that BMP-2 mRNA was hardly detected in control group. In induced group, the mRNA expression of BMP-2 was evaluated with the increase of UA concentrations ($P<0.05$). **Conclusions:** UA can upregulate expression of BMP-2 mRNA during osteogenic differentiation of hBMSCs.

Key words: UA; Bone marrow mesenchymal stem cells, human; Osteoblastic differentiation; Bone morphogenetic protein-2

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Introduction

Bone marrow mesenchymal stem cells (BMSCs) are multipotent cell that can be used as the seed cells in the bone tissue engineering. Nuttall's study [1]investigated that apparent reciprocal relationship exists between the adipogenesis and osteogenesis. In osteopenic disorders, osteogenic differentiation of BMSCs was inhibited with a concomitant increase in adipogenesis. Therefore, they believe that the inhibition of BMSCs adipogenesis and concomitan increase in osteogenesis may provide an approach to prevent or treat osteopenic disorders.

Osteoporosis (OP) is a disorder characterized by enhanced skeletal fragility due to a reduction in both bone quantity and quality. OP can cause fractures and mortality, especially in the elder, so study on OP got more and more attention all over the world. Many factors have effect on bone mineral density (BMD), such as body weight, blood glucose, blood lipid, et al. A longitudinal study [2] showed, serum uric acid (UA) plays a protective role for bone loss in peri- and postmenopausal women.

As the final breakdown product of purine metabolism, UA plays a dual role in the body. On the one hand, UA in excess may cause gouty arthritis and renal stones, on the other hand higher serum UA levels within normal physiologic levels may have conferred a selection advantage because of their antioxidant effects. A research has shown [3], UA can promote human bone marrow mesenchymal stem cells (hBMSCs) to proliferate and differentiate into osteoblasts with the increase of UA concentrations and time of induction, but the specific mechanism is unclear. Here, we observe the effect of UA on the expression of Bone morphogenetic protein-2 (BMP-2) in the process of osteogenesis differentiation in vitro.

1 Materials and methods

1.1 Materials

1.1.1 Source Bone marrow samples were separated from healthy volunteers aged 18 to 60. The volunteers were informed and the procedures were approved by the ethics committee of the Affiliated Hospital of Qingdao University.

1.1.2 Main reagent Low-glucose Dulbecco's modified Eagle's medium (L-DMEM), fetal bovine serum (FBS), trypsin

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Author introduction: XIE Ting-ting(1988-), female, studying for master's degree. Research field: Endocrine,

Tel: 18254250732, E-mail: zbxiaoxie@126.com

△Corresponding author: YANG Nai-long, E-mail: nailongy@163.com

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from Hyclone; Uric acid (UA) from Sigma,USA; dexamethasone, β-glycerol phosphatesodium, vitamin C from Solarbio, Beijing; Alkaline phosphatase assay kit (A059-2) from Nanjing Jiancheng Bioengineering Institute; total RNA kit I (R6834-01), M-MLY first strand cDNA synthesis kit (TQ2501-01), PCR amplification kit (P2011), 50bp ladder (M11-01) from from Omega, USA.

1.1.3 Main instrument Multi-function analyzer, CO_2 incubator, clean bench from Thermo, USA; inverted fluorescence microscope from Nikon, Japan; cell culture bottle and plate from Corning, USA.

1.2 Methods

1.2.1 Configuration of different culture medium Complete medium contained the volume fraction of 10 % FBS, 1 % double-antibiotics and 89 % L-DMEM. Osteogenic induction medium was composed of 10-8 mol/L dexamethasone, 10 mmol/L β-glycerol phosphatesodium, and 50 mg/L vitamin C in the complete medium. According to the method of Li Maoyue [4], 13.45 mg UA was dissolved in 1 mL of 1 mol/L NaOH solution till completely dissolved with an oscillation. Then, 0.5 mL solution was added into 49.5 mL complete medium (or osteogenic induction medium) with a pH value ranging 7.2-7.4 adjusted by 7% HCl. After filtration (0.22 μm), the complete medium (or osteogenic induction medium) of 0.8 mmol/L UA stored at a 4 ℃ refrigerator. When used, medium containing 0.8 mmol/L UA was diluted to the medium containing 0.4 mmol/L and 0.2 mmol/L UA.

1.2.2 Isolation culture and proliferation of hBMSCs 1 mL bone marrow from healthy volunteers was added into 4 mL complete medium into a 25 cm2 cell culture bottle and placed in a 5 % CO_2 incubator at 37 ℃. Semi-medium was changed after 3 days. The medium was removed 2-3 days later and replaced with the same volume of fresh medium. Cell morphology was observed through the inverted microscope. After reached 80 % confluence, the cells were passaged by 0.25 % trypsin and replated at 1:2-3 dilutions. Depending on the previous study [5], the cells were confirmed as hBMSCs.

1.2.3 Osteogenic differentiation of hBMSCs Passage 3 cells were seeded into 6-well plates at a density of 5×10^5 /mL. At 80 % confluence, the cells were divided into five groups and were transferred into osteogenic induction medium containing different concentrations of UA. The cells were cultured in the 5% CO_2 incubator at 37 ℃. Medium should be changed completely every 2-3 days. Cell morphology and change was observed through the inverted fluorescence microscope during the induced differentiation process.

1.2.4 Osteogenetic differentiation abilities test of hBMSCs Alizarin red staining After 14 days of induction, the cells were washed 3 times with PBS, fixed with 4 % paraformaldehyde for 10 min, washed 3 times with PBS, added into 1 % alizarin-Tris-HCL (pH 8.3), placed in the 37 ℃, 5 % CO_2 incubator for 30 minutes, washed 3 times with PBS, and were observed through the inverted microscope.

ALP activity assay The supernatant was removed on the 3rd, 6th, 9th, 12th, 14th day. The cells were washed 3 times with PBS, added into 0.1 % TritonX-100 500 μL, cracked completely for 40 min. The mix was collected, and the absorbance was measured on a multi-function analyzer with a test wavelength at 520 nm. Finally, ALP activity should be calculated according to the formula from the kit instruction.

1.2.5 Detection of the expression of BMP-2 mRNA After 14 days of osteogenic induction, total RNA in all groups were extracted with the E.Z.N.A.TM Total RNA Kit (Omega, USA). RNA underwent reverse transcription by using M-MLV frist strand cDNA Synthesis Kit (Omega, USA), and the resulting cDNA obtained was used for PCR amplification to estimate the expression of BMP-2. Glyceraldehyde phosphate dehydrogenase (GAPDH) was employed as an internal reference. The primer sequences used were: BMP-2 forward primer5'- GGTGGAATGACTGGATTG -3', reverse primer 5'- GCATCGAGATAGCACTG -3', yielding a 189 bp size product; and GAPDH forward primer 5'-GGTGGACCTGACCTGCCGTCTAGA-3',reverse primer 5'- TTACTCCTTGGAGGCCATGTGGG -3', yielding a 283 bp size product.

PCR amplification conditions are as follows: 5 min at 94 ℃ (pre-denaturation) followed by RT-PCR cycling of 30 s at 94 ℃ for denaturation, 30 s at 58 ℃ for annealing, and 60 s at 72 ℃ for extension, and lastly a final extension for 7 min at 72 ℃. RT-PCR was performed 35 cycles. RT-PCR products were combined and resolved in a 2 % agarose gel stained with GELVIEW. Documentation and semiquantitative evaluation was performed using a real-time camera combined with an Alpha-DigiDocTM RT software. The results for BMP-2 mRNA levels were presented relative to the expression of GAPDH.

1.3 Statistical analysis

All statistical calculations were performed with the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for statistical analysis, and data were expressed as mean± SD. Least significance difference test was utilized for comparison between two groups. $p<0.05$ was rated statistically significant and $P>0.05$ showed no significance. The average values (X) and standard deviations (SD) served as descriptive parameters.

2 Results

2.1 Cell morphology and change of primary and passage3 hBMSCs

Primary hBMSCs were grown adherently and first observed at 6th day (Fig. 1A). In the vision, adherent cells were scattered in polygons and irregular shape. With the extension of incubation

time, primary cells can passaged at 80 % confluence. Passage cells were single long-spindle cells and gradually formed a whirlpool-shaped arrangement after about 14 day (Fig. 1B).

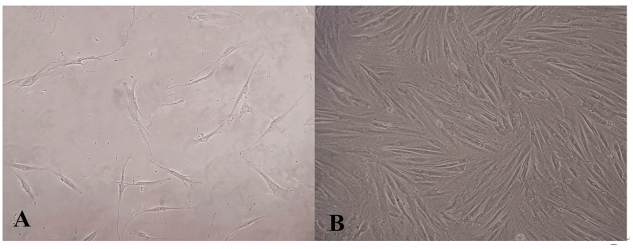

Fig. 1 Morphology of hBMSCs (× 100)

A: The 6th day of primary hBMSC; B: The 3rd generation of passaged hBMSC

2.2 Cell morphology and change of hBMSCs differentiating to osteoblasts

After osteogenic induction, the majority cells were from long-spindle to irregular cube in shape, and gradually became paving stone or formed nodules. With the increase of concentration of UA, cells were more intensive and increased in number. Among all groups, the cells in 0.8 mmol/L UA induction medium formed the most nodules (Fig. 2 ABCD).

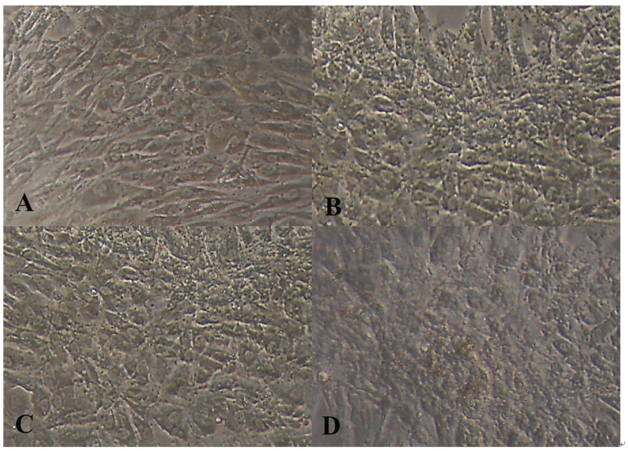

Fig. 2 Morphology of hBMSCs under different UA concentrations after 14 day induction (× 100)

A: 0 mmol/L UA; B: 0.2 mmol/L UA;

C: 0.4mmol/L UA; D: 0.8mmol/L UA

2.3 Osteogenic differentiation abilities test of hBMSCs

Alizarin red staining results showed, calcium nodules in the blank control group were negtive, however, calcium nodules were dyed orange in osteogenic induction group (Fig. 3A).

Alkaline phosphates (ALP) activity assay showed, ALP was hardly detected in the blank control group. With the increased of UA concentration and incubation time, ALP activities were increased gradually. ALP activity showed significant concentration-dependent and time-dependent (Fig. 3B, Table 1).

2.4 The expression of BMP-2 mRNA

RT-PCR results showed that BMP-2 mRNA hardly expressed in blank group. The ratio of BMP-2 compare to GAPDH in induced group was 0.325 ± 0.005, 0.670 ± 0.006, 0.778 ± 0.008, 0.985± 0.005. Compared with the osteogenesis induction medium containing 0 mmol/L UA, BMP-2 mRNA expression showed a concentration-dependent increase, and differences were considered significant ($P < 0.05$) (Fig. 4). The level of BMP-2 expression was highest in the osteogenesis induction medium containing 0.8 mmol/L UA.

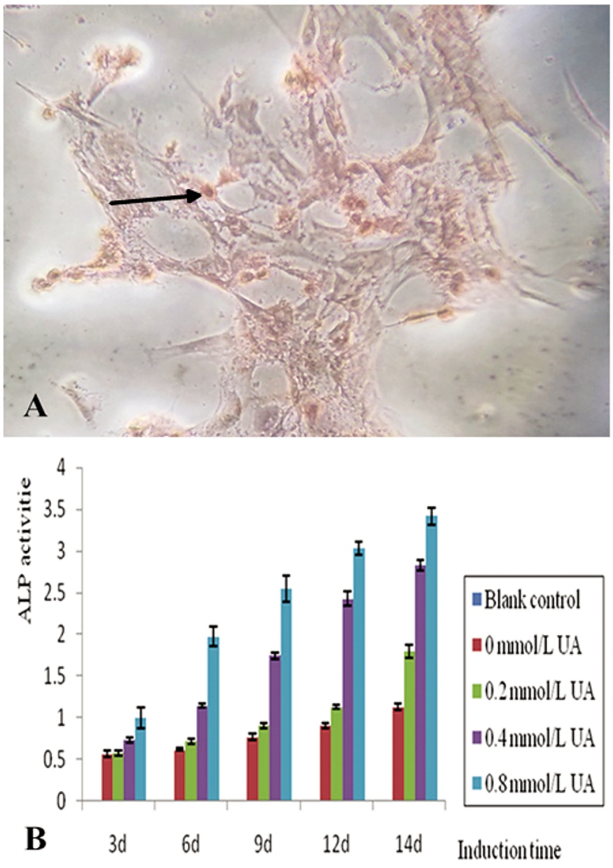

Fig. 3 Osteogenic differentiation abilities test of hBMSCs

A: Alizarin red staining (× 100); B: ALP activity and UA concentrations in different period

3 Discussion

UA is the end metabolic product of purine, mainly decomposed from nucleic acids and other purine compounds. Supranormal serum UA levels have been associated with presence of the metabolic syndrome[6], diabetes mellitus[7], cardiovascular disease[8], et al. Indeed, UA accounts for approximately half of the antioxidant properties of human plasma. Evidence from observational and epidemiological studies has linked oxidative stress or low circulating levels of anti-oxidants to reduced osteoporosis[9], improve cognitive function in the elderly [10], and improve the prognosis of Parkinson's disease and multiple sclerosis[11, 12].

BMSCs derived from bone marrow can be differentiated into

Table 1 ALP activity under different period and UA concentrations (King unit/100 mL) (X± s, n = 6)

	3d	6d	9d	12d	14d
Blank control	-	-	-	-	-
0 mM UA	0.5640± 0.0430	0.6160± 0.0168	0.7657± 0.0408^{d}	0.9049± 0.0268^{d}	1.1281± 0.0352^{de}
0.2 mM UA	0.5743± 0.0297	0.7151± 0.0308	0.9092± 0.0311^{ade}	1.1307± 0.0225^{ade}	1.7951± 0.0742^{adefg}
0.4 mM UA	0.7334± 0.0312^{a}	1.1480± 0.0190^{abd}	1.7441± 0.0356^{abde}	2.4296± 0.0856^{abdfe}	2.8215± 0.0634^{abdefg}
0.8 mM UA	0.9956± 0.1188^{abc}	1.9762± 0.1153^{abcd}	2.5405± 0.1569^{abcde}	3.0305± 0.0774^{abcdef}	3.4161± 0.0979^{abcdefg}

Notes: compared with the same period: ^{a}P <0.05, vs 0 mM UA; ^{b}P<0.05, vs 0.2 mM UA; ^{c}P<0.05, vs 0.4 mM UA; compared with the same UA concentrations: ^{d}P<0.05, vs the 3rd day; ^{e}P<0.05, vs the 6th day; ^{f}P<0.05, vs the 9th day; ^{g}P<0.05, vs the 12th day. (1 mM=1 mmol/L)

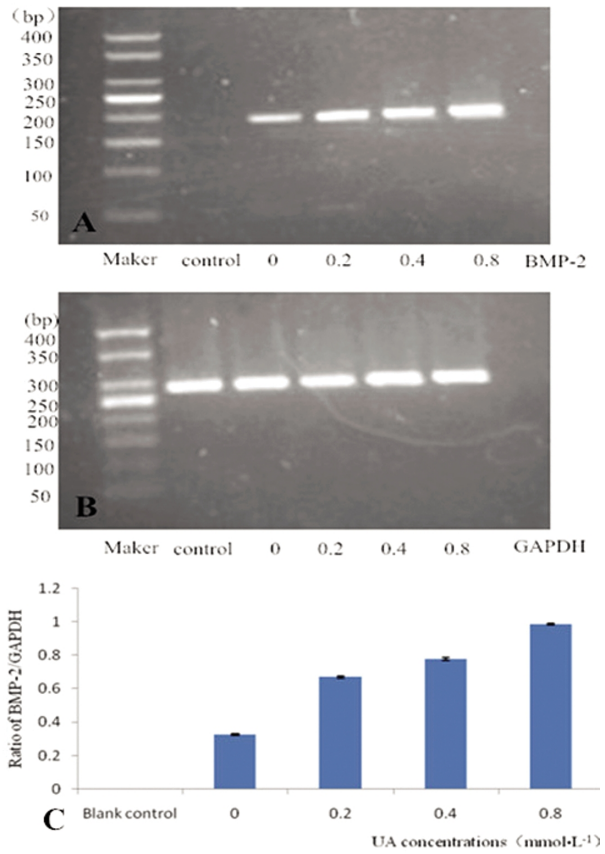

Fig. 4 BMP-2 mRNA expression of passage3 hBMSC induced for 14days
A: BMP-2 expression; B: GAPDH expression.
C: Ratio of BMP-2 compare to GAPDH at differrent UA concentrations

osteoblasts[13], myocardial cells[14], liver cells[15], nerve-like cells[16] in relatively simple induction conditions. This experiment above adopts joint induction containing 10^{-8} mol/L dexamethasone, 10 mmol/L β-glycerol phosphatesodium, 50 mg/L vitamin C. According the previous experimental result[17], we selected the osteogenic induction medium containing concentration of 0.2 mmol/L, 0.4 mmol/L and 0.8 mmol/L UA (physiological concentration of UA is 0.15-0.38 mmol/L in male and 0.1-0.3 mmol/L in female). During differentiation to osteoblast in vitro, osteoblast phenotypic markers appear in the following order: accumulation of collagenous matrix, expression of alkaline phosphatase, secretion of osteocalcin, and finally mineralization of bone nodules. ALP and mineralized nodule were chosen to represent the early and late evaluation index during osteogenesis[18]. After 14 days of induction, BMSCs were positive for calcium nodules by alizarin red, indicating that the cells have been induced to have osteogenic characteristics. With the increase of intervention time and UA concentration, ALP activity enhanced gradually. This suggests that UA can promote more BMSCs to differentiate into osteoblasts. In addition, proliferation and differentiation of BMSCs may be affected by osmotic pressure. But mannitol can't exist for a long time in the body, so we set no osmotic pressure of mannitol in the control group.

Bone morphogenetic proteins (BMPs) are multifunctional growth factors within the transforming growth factor β (TGF-β) super family that were identified based on their ability to initiate ectopic bone formation in adult animals. BMP-2 is considered to be one of the strongest osteogenic factors [19]. It not only can activate or enhance the expression of osteogenesis-related genes, such as [20, 21] OPN, Runx2 / Cbfal1, ALP, fatty acid coupling protein 4 (fabp4), but also can raise undifferentiated hBMSCs to differentiate into cartilage and osteoblast in bone matrix. However, BMP-2, as osteogenic promoter, has no effect on the differentiation of mature osteoblasts and chondrocytes. Wang and his co-workers [22] successfully induced BMSCs to differentiate into osteoblast and induced cells expressed BMP-2. The result of RT-PCR showed no BMP-2 mRNA expression in control group. Compared with induction medium containing 0 mmol/L UA, BMP-2 mRNA expression was respectively increased with the increase of UA concentration. It indicates that UA can promote BMP-2 mRNA expression, and BMP-2 promotes BMSCs to differentiating to osteoblast. The conclusion was UA can can upregulate expression of BMP-2 mRNA during osteogenic differentiation in a concentration-dependent manner.

BMSCs differentiating into osteoblast is extremely complicated progress, which containing interaction between BMP-2 and other regulatory factors. This study tries to reveal part mechanism of UA to promote osteogenesis, but we only observe the promotive effects of UA on osteogenic differentiation of BMSCs cultured in vitro during a short-term period, and cannot simulate the complex human system. More works need to determine the osteogenic mechanism and whether UA can prevent osteoporosis or not also

needs more study in the future.

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尿酸影响人骨髓间充质干细胞成骨分化过程中 BMP-2 表达 *

谢婷婷[1] 杨乃龙[1△] 徐丽丽[1] 王淑芳[2]

（1 青岛大学附属医院内分泌科 山东 青岛 266000；2 曹县人民医院 山东 菏泽 274000）

摘要 目的：探讨在人骨髓间充质干细胞（hBMSCs）成骨分化过程中，不同浓度尿酸（UA）对骨形态形成蛋白 -2（BMP-2）表达的影响。**方法：**以全骨髓贴壁培养法分离 hBMSCs，将生长状态良好的第 3 代 hBMSCs 分为 5 组，分别为空白对照组（加入完全培养基）和成骨诱导组（加入成骨诱导液及含 0 mmol/L、0.2 mmol/L、0.4 mmol/L、0.8 mmol/L 尿酸的完全培养基）。连续干预诱导 14d 后，用倒置显微镜观察细胞形态的变化，通过观察茜素红染色情况及检测碱性磷酸酶(ALP)活性进行成骨情况的检测。RT-PCR 技术检测各组细胞 BMP-2 mRNA 的表达情况。**结果：**第 3 代 hBMSCs 大多为形态单一的长梭形，呈旋涡状生长；干预诱导后的细胞逐渐变成不规则的立方形，局部形成团块状结节，以含尿酸浓度为 0.8 mmol/L 的成骨诱导培养基最为显著。连续干预 14d 后，空白对照组茜素红染色为阴性，而各成骨诱导组细胞茜素红染色结果为阳性，提示干预诱导后的细胞为成骨细胞。碱性磷酸酶活性随尿酸浓度的增加和干预时间的延长而增强（P<0.05）。RT-PCR 检测结果显示，空白对照组无 BMP-2 mRNA 的表达。成骨诱导组随培养基中尿酸浓度的增加，BMP-2 mRNA 表达逐渐增强，呈浓度依赖性（P < 0.05）。**结论：**尿酸上调 hBMSCs 向成骨细胞分化过程中 BMP-2 mRNA 的表达。

关键词：尿酸；人骨髓间充质干细胞；成骨分化；骨形态形成蛋白 -2

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作者简介：谢婷婷（1988-），女，硕士研究生，主要从事内分泌方向的研究，电话：18254250732，E-mail: zbxiaoxie@126.com

△通讯作者：杨乃龙，E-mail: nailongy@163.com

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