The expression of mRNA for peptidylarginine deiminase type 2 and type 4 in bone marrow CD34+ cells in rheumatoid arthritis

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Abstract

Objective

Antibodies directed to citrullinated proteins are highly specific for rheumatoid arthritis (RA). Citrullination is catalysed by peptidylarginine deiminase (PAD) enzymes. The current study examined the mRNA expression of PADI2 and PADI4 in bone marrow (BM) CD34+ cells from RA patients.

Methods

CD34+ cells were purified from BM samples obtained from 48 RA patients and from 30 osteoarthritis (OA) patients during joint operations via aspiration from the iliac crest. The expression of mRNAs for PADI2, PADI4 and Sp1 was examined by quantitative reverse transcription PCR.

Results

The expression of mRNA for PADI2 was significantly higher in RA BM CD34+ cells than OA BM CD34+ cells. The expression of mRNAs for PADI4 and Sp1 in RA BM CD34+ cells appeared to be increased compared to OA BM CD34+ cells, although it did not reach the statistical significance. The levels of mRNAs for PADI2, PADI4 and Sp1 were not correlated with serum C-reactive protein or with the administration of methotrexate or oral steroids. Finally, the level of PADI2 mRNA as well as that of PADI4 mRNA was significantly correlated with the level of Sp1 mRNA in RA BM CD34+ cells.

Conclusion

These results indicate that the mRNA expression of PADI2, PADI4 and Sp1 is upregulated in RA BM CD34+ cells independently of the systemic inflammation or treatment regimen. Moreover, the data suggest that the enhanced mRNA expression of PADI2 and PADI4 in BM CD34+ cells might be a result of the enhanced expression of Sp1 gene in RA BM CD34+ cells.

Key words

rheumatoid arthritis, bone marrow, CD34, PADI, Sp1

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by hyperplasia of synovial lining cells, consisting of macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes (1). Type A synoviocytes, also called intimal macrophages, have been shown to be derived from monocyte precursors in the bone marrow (BM) (2, 3). On the other hand, type B synoviocytes, also called fibroblast-like synoviocytes, have the morphologic appearance of fibroblasts as well as the capacity to produce and secrete a variety of factors, including proteoglycans, cytokines, arachidonic acid metabolites, and matrix metalloproteinases (MMPs), and play an important role in the destruction of joints (1). Although the precise origin of type B synoviocytes has been unclear (4), accumulating studies have disclosed that type B synoviocytes are also derived from BM. Thus, it has been demonstrated that BM $CD34^+$ cells from RA patients have abnormal capacities to respond to tumour necrosis factor-α (TNF-α) and to differentiate into fibroblast-like cells producing MMP-1, suggesting that BM $CD34^+$ progenitor cells might generate type B synoviocytes and thus could play an important role in the pathogenesis of RA (5).

Anti-citrullinated protein/peptide antibodies (ACPA) are highly specific for RA (6, 7). Of note, it has been recently shown that ACPA titers increased markedly a few years before the development of RA (8, 9). Citrullination is catalysed by a group of peptidylarginine deiminase (PAD) enzymes including five isoforms (PAD1, 2, 3, 4 and 6) with differential cellular and tissue distribution (10). Previous studies have disclosed that PADI4 gene polymorphisms are associated with RA in East Asian populations, providing evidence for the involvement of PADI4 in the pathogenesis of RA (7, 11, 12).

Human PAD4 is expressed in haematopoietic cells, whereas human PAD2 is present in a broad range of tissues, including skeletal muscle, uterus, brain, salivary glands and pancreas (10). Of note, PAD2 protein has been detected in *ex vivo* differentiated macrophages and mononuclear cells from synovial fluid of RA patients, suggesting that PADI2 also plays an important role in the pathogenesis of RA (13). Moreover, a recent report revealed a significant association of three PADI2 SNPs with RA in the Shandong area of Northern China (14).

Although the transcriptional regulation of PADI genes has not been fully investigated, recent studies have found that Sp1 transcriptional factor binds to the promoter of PADI2 and PADI4 (15, 16). On the other hand, previous studies have demonstrated the abnormal expression of mRNAs for several genes, including nuclear factor kappaB (NF-κB) 1, Krüppel-like factor 5 (KLF5), FKBP5 and Flt3 ligand in BM $CD34^+$ cells in RA (17-20). It is thus suggested that BM $CD34^+$ cells might also have abnormal expression of PADI2 and PADI4 genes. The current study therefore examined the mRNA expression of PADI2, PADI4 and Sp1 in BM $CD34^+$ cells from RA patients.

Materials and methods

Patients and samples

BM samples were obtained from 48 patients with RA (6 males and 42 females: mean age, 58.8 years; age range, 35 to 78 years) who satisfied the American College of Rheumatology 1987 revised criteria for RA (21) and gave informed consent in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. This study was conducted with the approval of Osaka University Research Ethics Committee. The approval number of the approving board is "10246-2". 5 to 10 ml of the samples was taken during joint operations via aspiration from the iliac crest under anesthesia. As a control, BM samples were similarly obtained from 30 patients with osteoarthritis (OA) (3 males and 27 females; mean age, 71.1 years; age range, 49 to 81 years) who gave informed consent. Most patients with RA and OA were taking non-steroidal anti-inflammatory drugs. Of the 48 patients with RA, 26 were treated with low dose methotrexate (MTX) and 36 were taking oral steroids when BM samples

were obtained. No OA patients were taking MTX or oral steroids.

Preparation of BM CD34⁺ cells

Mononuclear cells were isolated by centrifugation of heparinised BM aspirates over sodium diatrizoate-Ficoll gradients. CD34⁺ cells were purified from the mononuclear cells by positive selection with magnetic beads (CD34 progenitor cell selection system; Dynal, Oslo, Norway). The cells thus prepared were >95% CD34⁺ cells, <0.5% CD14⁺ monocytes, <0.5% CD3⁺ T cells and <0.5% CD19⁺ B cells, as previously described (5).

RNA isolation and real-time quantitative PCR

Total RNA was isolated from purified BM CD34⁺ cells using the Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. cDNA samples were prepared from 1 μg of total RNA using the SuperScript reverse transcriptase preamplification system (Life Technologies) with oligo (dT) primer and subjected to PCR. Real-time quantitative PCR was performed using LightCycler 1.5 Instrument (Roche Diagnostics, Mannheim, Germany) with primer sets for PADI2 (HA154112, Takara Bio, Otsu, Japan), PADI4 (HA186901, Takara Bio), Sp1 (HA145267, Takara Bio) or β-actin and SYBR *Premix Ex Taq*™ II (Takara Bio). The detail of primer sequences is shown in Table I. Quantitative analysis was performed using LightCycler Software v. 4.1. PCR reaction conditions composed of denaturing at 95°C for 30 seconds, followed by 45 PCR cycles (95°C for 5 seconds and 60°C for 20 seconds) for PADI2, PADI4, and Sp1, or 40 PCR cycles (95°C for 10 seconds, 62°C for 10 seconds and 72°C for 10 seconds) for β-actin. The data are expressed as the ratio of the mRNA copy numbers for PADI2, PADI4 or Sp1 to those of β-actin.

Culture medium, cytokines and stimulation of BM CD34⁺ cells from healthy individuals

RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with L-glutamine (0.3 mg/ml) and 10% fetal bovine serum (Life Technologies) was used for all cultures. Recombinant human TNF-α was purchased from Pepro Tech EC, London, UK. Highly purified BM CD34⁺ cells (5 x 10^5/well) from healthy individuals (BioWhittaker, Walkersville, MD) were cultured in 24-well microtiter plates (Nunc, Roskilde, Denmark) in the presence of TNF-α (10 ng/ml) for 24 to 72 h. After the cultures, the expression of mRNAs for PADI2, PADI4 and Sp1 was examined, as mentioned above.

Statistical analysis

The results were analysed for statistical significance by Mann Whitney U-test, or Spearman's rank correlation test, where appropriate.

Table I. Primer sequences used in real-time quantitative PCR.

Gene product (Genbank Accession No.)	Primer sequences	Nucleotides
PADI2 (NM007365.2)	Forward: 5'-GTGACAACCCTCGGTGTGGA-3'	4076-4095
	Reverse: 5'-ACATCAAGGTGGAAGCAGGAACTTA-3'	4183-4207
PADI4 (NM012387.2)	Forward: 5'-TGAAAGCCAAGTGCAAGCTGA-3'	1011-1031
	Reverse: 5'-GGCTTGGATGTAGCCGATCTC-3'	1085-1105
Sp1 (NM138473.2)	Forward: 5'-GACTAGGGCTGCAAGTAGTGAGGA-3'	6890-6913
	Reverse: 5'-GGAAACTGGAGCACTGGGTAGAC-3'	7003-7025
β-actin (NM001101.3)	Forward: 5'-GCAAAGACCTGTACGCCAAC-3'	953- 972
	Reverse: 5'-CTAGAAGCATTTGCGGTGGA-3'	1193-1212

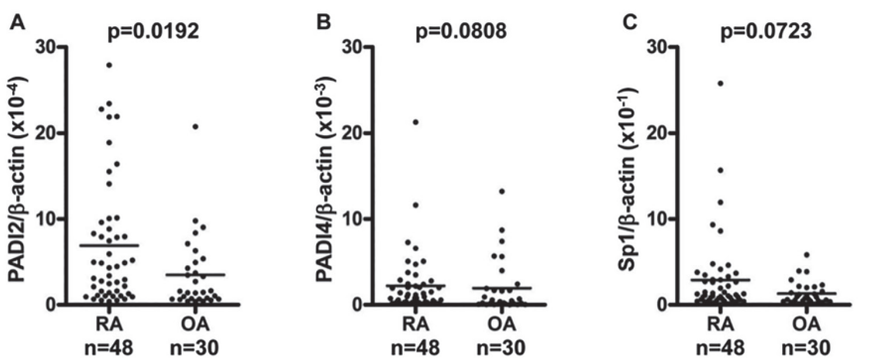

Fig. 1. Quantitative analysis of the expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34+ cells. Total RNA was isolated from purified BM CD34+ cells from 48 RA patients and 30 OA patients. The expression of mRNAs for PADI2, PADI4, Sp1 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers for PADI2, PADI4 or Sp1 to those of β-actin. Horizontal lines indicate the mean values. Statistical analysis was performed by Mann Whitney U-test.

Results

Expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34⁺ cells

Initial experiments compared the levels of mRNAs for PADI2, PADI4 and Sp1 in RA BM CD34⁺ cells with those in OA BM CD34⁺ cells. The expression of PADI2 mRNA was significantly higher in RA BM CD34⁺ cells than in OA BM CD34⁺ cells (p=0.0192, Fig. 1A). The expression of mRNAs for PADI4 and Sp1 appeared to be higher in RA BM CD34⁺ cells than in OA BM CD34⁺ cells, although it did not reach the statistical significance (p=0.0808 and p=0.0723, respectively, Figure 1B and 1C). As shown in Figure 2, the mRNA expression of Sp1 was significantly correlated with that of PADI2 or with that of PADI4 in RA BM CD34⁺ cells (r=0.7954, p<0.0001 and r=0.5180, p=0.0004, respectively). The mRNA expression of PADI2 was also significantly correlated with that of PADI4 in RA BM CD34⁺ cells (r=0.7143, p<0.0001). The data therefore suggest that the upregulation of mRNAs for PADI2 and PADI4 might be resulted from the enhanced expression of Sp1 mRNA in RA BM CD34⁺ cells.

Of note, 26 and 36 of the 48 RA pa-

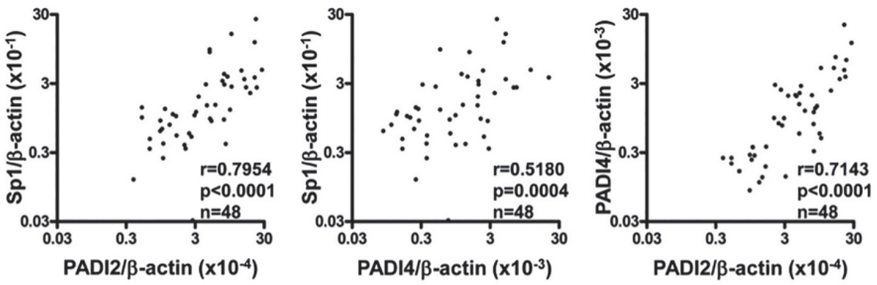

Fig. 2. Correlation of mRNAs for PADI2 and PADI4 with Sp1 mRNA in BM CD34+ cells. Total RNA was isolated from purified BM CD34+ cells from 48 RA patients. The expression of mRNAs for PADI2, PADI4, Sp1 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers for PADI2, PADI4 or Sp1 to those of β-actin. Statistical significance was evaluated by Spearman's rank correlation test.

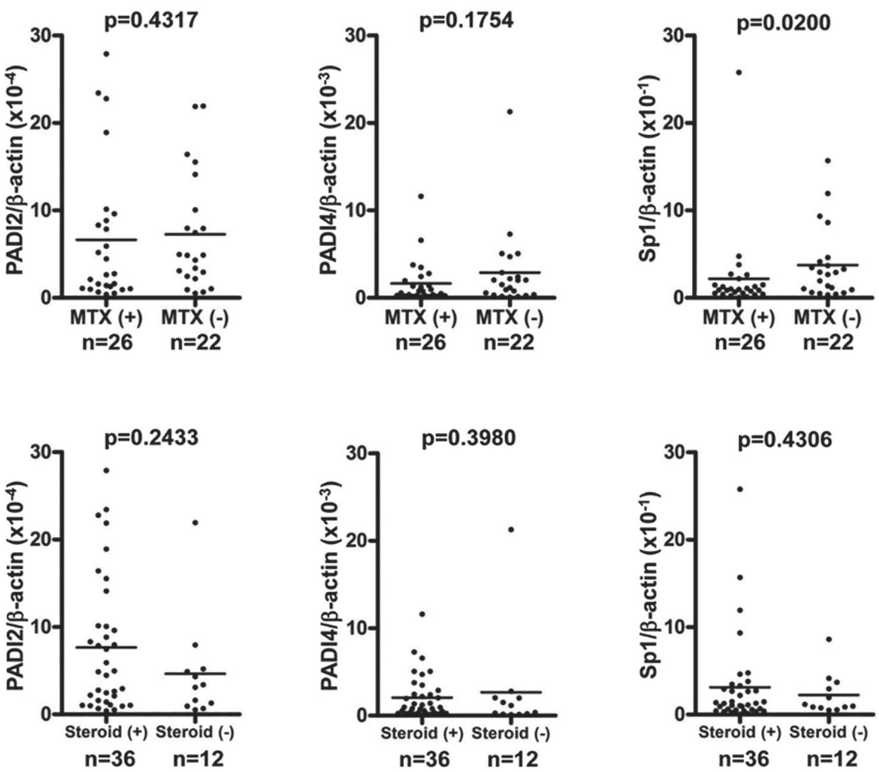

Fig. 3. The relevance of treatment with the expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34+ cells. Total RNA was isolated from purified BM CD34+ cells from 48 RA patients. The expression of mRNAs for PADI2, PADI4, Sp1 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers for PADI2, PADI4 or Sp1 to those of β-actin. Effect of treatment with methotrexate (MTX) (upper panel) or oral steroids (Steroid) (lower panel) was evaluated by Mann-Whitney U-test. Horizontal lines and whiskers indicate the mean values.

tients were treated with MTX and oral steroids, respectively, whereas no OA patients were taking either MTX or oral steroids. It is therefore possible that MTX and oral steroids might have affected the expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34$^+$ cells. As shown in Figure 3, however, there were no significant differences in the expression of mRNAs for PADI2 and PADI4 in BM CD34$^+$ cells between RA patients taking MTX and those not taking MTX, whereas the expression of Sp1 mRNA was significantly lower in RA patients taking MTX. The expression of mRNAs for all the 3 genes was not different between RA patients with oral steroids and those without oral steroids. It should be also noted that the expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34$^+$ cells was not significantly correlated with serum C-reactive protein (CRP) levels in RA patients (Fig. 4). The data thus indicate that the expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34$^+$ cells is upregulated independent of the activity of systemic inflammation or treatment regimens.

Lack of enhancement of mRNAs for PADI2, PADI4 and Sp1 in normal BM CD34$^+$ cells by TNF-α

Previous studies have demonstrated that TNF-α plays a critical role in the pathogenesis of RA (4). It is therefore possible that the up-regulation of mRNAs for PADI2, PADI4 and Sp1 in BM CD34$^+$ cells might be secondary to the action of TNF-α. Next experiments were therefore carried out to test this possibility. As shown in Table II, treatment of BM CD34$^+$ cells with TNF-α did not upregulate the expression of mRNAs for PADI2, PADI4 or Sp1, but rather decreased it. These results indicate that the increased expression of mRNAs for PADI2, PADI4 and Sp1 in RA BM CD34$^+$ cells is not accounted for by the action of TNF-α, but possibly by intrinsic abnormalities of BM CD34$^+$ cells.

Discussion

Among five PAD enzymes, only PAD2 and PAD4 have been identified in the synovium of RA patients (22). Our data showed that the expression of mRNAs for PADI2 and PADI4 was enhanced in RA BM CD34$^+$ cells. Several studies have disclosed that both type A synoviocytes and type B synoviocytes are derived from BM (2, 3, 5). It is therefore suggested that the upregulation of mRNAs for PADI2 and PADI4 in RA BM CD34$^+$ cells might lead to the enhanced expression of PAD2 and PAD4 in RA synovial tissue.

Although PAD2 and PAD4 could be involved in citrullination of various proteins in RA synovium, several studies have also delineated that PAD2 and PAD4 plays important roles in the function of monocytes. Thus, the numbers of myeloid lineage cells, such as neutrophils and monocytes, were decreased in glucose-6-phosphate isomerase-immunised PADI4-deficient mice, suggesting that PADI4 controls the survival of myeloid lineage cells (23). On the other hand, PAD2-overex-

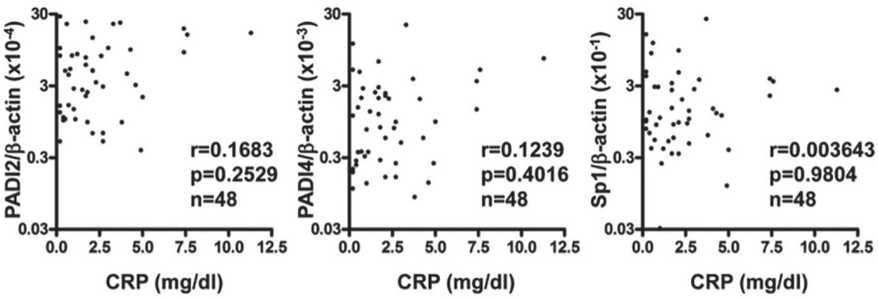

Fig. 4. Correlation of mRNAs for PADI2, PADI4 and Sp1 in BM CD34+ cells with serum CRP in RA patients. Total RNA was isolated from purified BM CD34+ cells from 48 RA patients. The expression of mRNAs for PADI2, PADI4, Sp1 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers for PADI2, PADI4 or Sp1 to those of β-actin. Statistical significance was evaluated by Spearman's rank correlation test.

Table II. Effect of tumour necrosis factor (TNF)--α on the expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34+ cells.

Expt. (Time)	Addition	PADI2/β-actin (x10^{-4})	PADI4/β-actin (x10^{-3})	Sp1/β-actin (x10^{-1})
1 (24h)	Nil	31.2	8.56	26.3
	TNF-α	7.90	0.08	6.56
2 (24h)	Nil	45.7	3.34	27.1
	TNF-α	8.70	0.37	8.06
3 (24h)	Nil	37.9	0.384	47.7
	TNF-α	32.8	0.0256	49.6
3 (72h)	Nil	56.1	0.0112	33.2
	TNF-α	41.1	<0.0028	26.8

BM CD34+ cells from healthy individuals were incubated with or without TNF-α (10 ng/ml) for 24 to 72h. After the incubation, total RNA was isolated for evaluation of the expression of mRNAs for PADI2, PADI4, Sp1 and β-actin by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers for PADI2, PADI4 or Sp1 to those of β-actin.

pressing human A431 cells increased the expression of mRNAs for IL-6 and IL-8 as well as that of mRNAs for inflammatory mediators NF-κB/RelA, thus promoting inflammatory responses (24). Previous studies have disclosed that RA BM CD34+ cells have abnormal capacities to respond to TNF-α (5), and that the enhanced expression of NF-κB1 mRNA in RA RM CD34+ cells is important for the enhanced generation of type B synoviocytes (17). It is therefore possible that the enhanced expression of mRNAs for PADI2 and PADI4 in RA BM CD34+ cells might play a pivotal role in the pathogenesis of RA by altering the function of monocytes or macrophages.

Since there was no significant correlation between the expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34+ cells and serum CRP, the enhanced expression of mRNAs for these three genes in BM CD34+ cells might not be a result of systemic inflammation. Of note, a number of studies have shown that TNF-α plays a pivotal role in the pathogenesis of RA (25-27). However, TNF-α did not increase the expression of mRNAs for PADI2, PADI4 or Sp1 in BM CD34+ cells from normal healthy individuals. Taken together, these results suggest that the enhanced expression of mRNAs for PADI2, PADI4 and Sp1 in BM CD34+ cells might not be secondary to inflammatory process of RA, but rather be a primary intrinsic abnormality in RA. MTX reduced the expression of Sp1 mRNA in RA BM CD34+ cells. Therefore, it is likely that the level of Sp1 mRNA in BM CD34+ cells of untreated or MTX-naïve RA patients might be much more higher than that in BM CD34+ cells from RA patients in the present study.

Previous studies have disclosed that Sp1 is involved in the transcriptional regulation of PADI2 and PADI4 (15, 16). It is therefore suggested that the abnormal expression of Sp1 mRNA in RA BM CD34+ cells might enhance the transcription of these two PADI genes, thus leading to the abnormal function of macrophages in RA. Of note, PADI4 gene polymorphisms have been found to be associated with RA in East Asian populations (7, 11, 12), whereas genome wide association studies have identified no significant relationship between PADI2 gene and RA. It is therefore suggested that the upregulation of mRNAs for PADI4 and PADI2 might not be related with the polymorphisms of PADI4 or PADI2. Rather, the data strongly suggest that the upregulation of Sp1 mRNA might play a pivotal role in the enhanced expression of mRNAs for PADI4 and PADI2.

We previously demonstrated the enhanced expression of KLF5 mRNA in RA BM CD34+ cells (18). Several studies have shown that Sp1 is involved in the transcriptional regulation of KLF5, also known as BTEB2 (28, 29). Thus, the upregulation of Sp1 mRNA in RA BM CD34+ cells could induce the enhanced expression of KLF5 mRNA as well as that of mRNAs for PADI4 and PADI2 in RA BM CD34+ cells. The mechanism for the upregulation of Sp1 mRNA in RA BM CD34+ cells remains unclear, although it is strongly suggested that Sp1 might be an upstream regulator of mRNAs for PADI2, PADI4 and KLF5 in RA BM CD34+ cells. Therefore, further studies to explore the mechanism of abnormal expression of Sp1 mRNA in RA BM CD34+ cells would be important for our complete understanding of the pathogenesis as well as the aetiology of RA.

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