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Biology of bone morphogenetic protein in bone repair and regeneration: A role for autologous blood coagulum as carrier

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ABSTRACT

BMPs were purified from demineralized bone matrix based on their ability to induce new bone *in vivo* and they represent a large member of the TGF-β superfamily of proteins. BMPs serve as morphogenic signals for mesenchymal stem cell migration, proliferation and subsequently differentiation into cartilage and bone during embryonic development. A BMP when implanted with a collagenous carrier in a rat subcutaneous site is capable of inducing new bone by mimicking the cellular events of embryonic bone formation. Based on this biological principle, BMP2 and BMP7 containing collagenous matrix as carrier have been developed as bone graft substitutes for spine fusion and long bone fractures. Here, we describe a novel autologous bone graft substitute that contains BMP6 delivered within an autologous blood coagulum as carrier and summarize the biology of osteogenic BMPs in the context of bone repair and regeneration specifically the critical role that carrier plays to support osteogenesis.

1. BMPs during embryonic skeletal development

BMPs are potent chemo-attractants [1,2], mitogens [3] and morphogens [4–7], and act across a concentration gradient during embryonic skeletal development [8,9]. BMPs recruit mesenchymal stem cells and promote condensation (proliferation) and subsequently trigger their differentiation into endochondral bone during skeletal morphogenesis [10,11]. Ectoderm generally expresses BMPs [12,13] as secretary proteins, which bind to extracellular matrix proteins (*e.g.*, heparin sulfate proteoglycans and type IV collagen), BMP-antagonists (Noggin, Chordin, Sclerostin, Gremlins) and are subsequently released and governed as needed for mesoderm condensation and differentiation [8,14,15]. The cells that express BMPs also express BMP antagonists in order to establish a concentration gradient for ligand-receptor interactions to induce the downstream signaling [9,16]. BMP signals are tightly controlled in space and time and the loss of an osteogenic BMP function at given tissue compartment is compensated by another BMP. Furthermore, BMP-signaling cross talks with TGF-β and activin as well as other members of the TGF-β superfamily proteins, and with Wnt- and Hedgehog-signals to govern skeletal tissue morphogenesis [16–18].

The embryonic cellular events that culminate in the formation of new cartilage and bone can be recapitulated in post-fetal life by implanting an osteogenic BMP (*e.g.*, BMP2, BMP4, BMP6 and BMP7) with a carrier in a rat subcutaneous site and in diaphyseal fracture, segmental defect and lumbar spine fusion models. The presence of BMP in the implant attracts a sufficient number of mesenchymal stem cells, induces proliferation and differentiation into bone [19–21]. This biological function of BMP is concentration-dependent, the lower the amount is motogenic (chemotaxis) and medium concentrations are mitogenic (proliferation) and higher concentrations are morphogenic (differentiation) [10,13,22,23]. The biological activities of BMPs with respect to chemotaxis, proliferation and differentiation have been demonstrated *in vitro* using Boyden chamber assay, cell proliferation and differentiation assays in cultures using a BMP and responding mesenchymal stem cells [1,24].

2. BMP structure and receptors

BMPs are homodimers and all have the hallmark of “7- cysteine domain” held by an inter-disulfide bridge at the 4th cysteine between two monomers and are highly conserved from fly to humans. BMPs are produced as a large precursor with signal peptide, pro-domain and mature “7-cystein TGF-β domain”. They are synthesized as a monomer with three intra-disulfide bridges and then undergo dimerization in the endoplasmic reticulum by forming inter-disulfide bridge at the 4th cysteine and processing at RXXR site before they are secreted into the extracellular space [25,26]. The secreted BMP protein is a dimer at the mature TGF-β domain, which is biologically active, whereas the pro-

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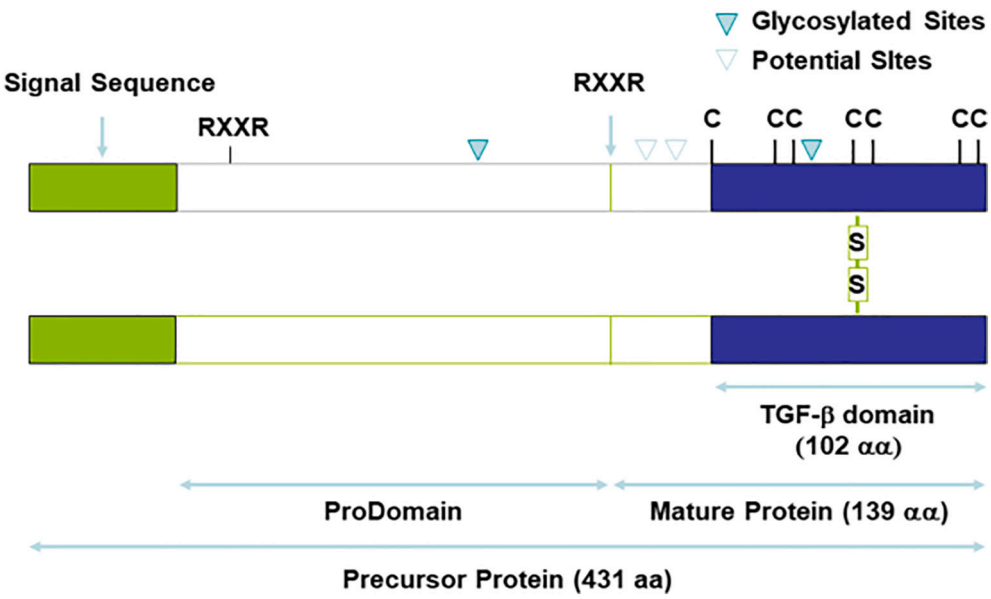

Fig. 1. Structure of BMP7/OP1.

domain is not active but can interact with mature, processed dimer by non-covalent interactions. The mature protein loses its biological activity if the inter-disulfide bridge is broken. The crystal structure reveals that the BMP dimer is aligned antiparallel with finger 1 and finger 2 and heal region [27]. A cysteine knot with intra- and inter- disulfide bridges holds the dimer protein and because of this it is very stable, even against proteases like trypsin. As an example, a schematic diagram for the structure of BMP7/OP1 is presented in Fig. 1.

BMP7 protein is composed of 431 amino acids that contains a 29 amino acids signal peptide, a 29–292 amino acid pro-domain and is cleaved at the second RXXR site to release 293–431 amino acids as processed mature protein containing a 7-cysteine domain, a hallmark of TGF-beta family of proteins. Though it has 4-potential glycosylation sites (marked as triangles) only two sites are glycosylated. However, the glycosylation is not required for bone induction.

BMPs signal through Ser-Thr kinase receptors type I and type II. Although both type I and type II bind to the ligand and form a complex, type I receptor renders specificity and recruits intracellular signaling kinases SMAD-1/5/8 and subsequently triggers phosphorylation. These SMADs complex with a co-SMAD-4 translocate into nucleus to switch-on and off sets of genes responsible for tissue morphogenesis, repair and regeneration [28]. A BMP employs a specific type I receptor (Activin Like Kinases, ALK-2 or ALK-3 or ALK-6;) and a type II receptor (BMPRII, ActRII-A and ActRII-B) depending on the cell type and cellular responses it triggers [29]. There are several BMP co-receptors that have been described to activate or inhibit BMP-signaling to trigger specific cellular function and outcome [30]. These include the Dragon family of proteins, Hemojuvelin and Endoglin. Two downstream inhibitors, SMAD-6 and -7 are identified to play a functional role as checkpoints by inhibiting the BMP downstream signaling to modulate the biological activity. BMP ligands can also trigger SMAD independent non-canonical downstream signaling directly or indirectly, such as MAPK/ERK/JNK/p38/PI3K/Akt/RANK/RANKL, as well as substantial cross-talk with the Wnt, hedgehog and VEGF signaling cascades [10,14]. In addition, known BMP antagonists like Noggin, Chordin, Follistatin, Gremlin, Sclerostin and USAG-1 are shown to govern the availability of BMP ligand to its receptor by binding avidly at the extracellular space to render specificity and establish a concentration gradient [31].

3. *In vitro* and *in vivo* model systems for endochondral bone formation

3.1. *In vitro* model systems

Several *in vitro* cell cultures have been used to examine BMP-like activity. Primary cultures generated from chick [32,33] and mouse-limb-bud [34], synovial tissue [35], skeletal muscle [36], periosteum [37,38], vasculatures [39] and primary bovine articular chondrocytes [40,41] and calvarial-derived primary osteoblasts [21,42] and established rat osteosarcoma cell lines [43], C2C12 mouse myoblast cell line [44], bone marrow derived W-29 stromal cells [45] and adipocytes [46] have been routinely employed. To examine mesenchymal stem cell differentiation into chondrocytes or osteoblasts, the early responsive genes like *id*-1, -2 and -3 were examined [47], for chondrogenic differentiation determinants like Sox-5, -9 [48] and markers of chondrocyte phenotype, type II collagen and cartilage-specific proteoglycan [51] were examined, for osteoblast differentiation, determinants like Osterix and Runx2 [49,50], and markers of osteoblast phenotype, alkaline phosphatase and osteocalcin are routinely monitored [21]. Identification of BMP-Responding Elements (BRE) in the promotor region of the BMP-SMAD dependent responding genes has allowed the engineering of several established stable cell lines linking with luciferase enzyme to specifically qualify the biological activity of BMP from cell- and tissue- extracts, body fluids, and for release assays for recombinant BMP production [52]. Furthermore, pluripotent stem cells generated from patients with musculoskeletal disorders are being employed to determine how BMPs drive chondrogenesis and osteogenesis using the loss or gain of function approaches and by establishing screens to select small molecules [53].

3.2. *In vivo* model systems

A BMP alone when implanted with an appropriate collagenous matrix can induce new bone formation at ectopic or orthotopic sites. This serves as a prototype for tissue engineering [54]. BMP serves as signal and collagen serves as scaffold. The local implant site provides a microenvironment to recruit the responding cells and they attach onto the collagenous scaffold and promote the differentiation into endochondral bone. This BMP-induced new bone formation is dose-dependent [21] up to certain doses based on given substratum used; however, at a higher dose BMP can trigger more recruitment and

proliferation of progenitors, resulting in cyst-like condensation and a delay in the differentiation into bone. This high dose cyst-phenomenon is observed both in ectopic and orthotopic sites. Recently, significant information about BMPs in systemic bone volume and heterotopic bone formation has been explored in genetically modified mice [26,55].

3.3. Role of BMPs in bone repair and regeneration

Several clinical trials have been conducted to assess the safety and efficacy of recombinant human BMP containing osteogenic devices for the treatment of acute diaphysis bone fractures and delayed union, tibial non-union and for anterior lumbar interbody fusion (ALIF) and posterolateral lumbar (PLF) fusion. Two BMP products, rhBMP2 (InFUSE®) [56] and rhBMP7 (OP-1® [57] and OP-1 Putty®) [58] are licensed under PMA and HDE for marketing and clinical application in the US [59–61].

OP-1® Implant: The first human clinical study was performed to assess the efficacy of recombinant human rhBMP7 (OP-1®) for the treatment of tibial non-union in a prospective, randomized and controlled clinical trial [62]. The conclusion of this clinical study demonstrated that OP-1® implant was safe and an effective treatment modality for a tibial non-union, and the outcome was comparable to the use of bone autograft but failed to achieve a significant difference as the number of patients included in the study were not sufficient, because of this it has received only HDE approval in the US.

OP-1 Putty®: OP-1® Implant was used in conjunction with carboxymethylcellulose to provide putty-like property. The OP-1 Putty® device was evaluated in the PLF clinical study to treat symptomatic single-level degenerative lumbar spondylolisthesis and spinal stenosis without instrumentation [63,64]. Outcomes measured at 12 months of the follow-up showed a positive trend but did not again meet a significant difference. Therefore, OP-1 Putty® received again HDE approval for use as an alternative to autograft in compromised patients requiring revision of the posterolateral (inter-transverse) lumbar spinal fusion.

InFUSE® (rhBMP2) was approved by FDA *via* premarketing approval (PMA) process, in conjunction with Absorbable Collagen Sponge (ACS) and LT-Cage Lumbar Tapered Fusion device for spinal fusion procedures *via* an anterior approach; the specific indication is for spinal fusion procedures in skeletally mature patients with degenerative disc disease (DDD) at one level from L2-S1 [65–67]. However, in large clinical studies conducted using a high dose (40 mg/single-level fusion) of InFUSE® with osteoconductive bulking agents (Amplify™) did not result in a positive outcome against autologous ICBG used as a comparator [68,69].

The off-label use of InFUSE® in cervical spine fusion posed unwanted safety issues including swelling of neck and throat tissue, which resulted in compression of the airway and/or neurological structures in the neck [70]. Some reports described difficulty in swallowing, breathing or speaking. Though fewer documented adverse events can be attributed to BMP, certain complications and safety issues are of concern. Adverse events that have been reported include but are not limited to inflammation, unwanted ectopic bone formation, infection, immune responses, vertebral osteolysis and vertebral edema. The concern is centered on excessive dose of BMPs (for example hrBMP2 applied 12–40 mg for a single-level fusion), the use of animal-sourced collagen (bovine type I collagen) and synthetic ceramics (hydroxyapatite and tri-calcium phosphate) composite as substratum to deliver rhBMP2 at the implant site [71,72]. Animal sourced collagens, ceramics as carriers induce inflammatory cytokine release and immune reactions at the local implant sites. Lower doses of BMPs with an appropriate physiological autologous scaffold might provide the optimal bone formation without provoking unwanted ectopic bone formation.

4. Autologous bone graft substitute – RhBMP6 in autologous blood coagulum

The most important component in BMP-based osteogenic device is the carrier/scaffold. The current BMP2 containing osteogenic device (inFuse®) utilizes bovine-derived collagen by alone or in combination with ceramics (hydroxyapatite and tri-calcium phosphate). The animal-derived collagen in the BMP2 device triggers initially immune responses and promotes the expression of markers associated with the fibroblast phenotype, and collagen-ceramics provokes inflammation and foreign body reaction. In addition, because of its low affinity to collagen/ceramics BMP2 is diffused out readily from the implant site and induces unwanted ossification at the distant sites. In order to overcome these unwanted inflammation and immune responses and compensate the immediate surge of BMP2 from the implant site, high doses of BMP2 (12–40 mg) are employed in the current osteogenic device. This safety issues observed in the clinical studies for posterolateral fusion have been ascribed to a high dose of BMP2 and the use of animal-derived collagen in combination and high mineral containing ceramic composites. In situation where the site is compromised due to non-union as seen in tibial diaphysis where the responding cells are not readily available in sufficient quantity, therefore autologous bone marrow is supplemented with InFuse® (BMP2 containing collagen scaffold).

A preferred scaffold for BMP would be an autologous physiological carrier which does not provoke inflammatory and immune responses like animal (bovine) derived collagen and exhibits a high affinity for BMPs. Hence, low doses of BMPs could be employed to induce bone formation without causing any unwanted safety concerns. We recently described a novel autologous bone graft substitute (ABGS) that contains recombinant human BMP6 delivered in autologous blood coagulum (ABC) which serves as a physiological carrier. ABGS was capable of inducing new bone formation in a rat subcutaneous site and repairing diaphyseal segmental defect in rabbits [73], as well as promoting posterolateral lumbar fusion (PLF) in rabbits [74] and sheep [75] models at low doses. Fig. 2 shows endochondral bone differentiation in the rat subcutaneous implants induced by ABGS (rhBMP6/ABC) without and with rat bone allograft used as compression resistant matrix (CRM). ABGS induces a cascade of cellular events leading to endochondral ossification resulting in cartilage (day 7), bone (day 14) bone and bone marrow (day 35) in a reproducible manner. On the other hand, in the absence of rhBMP6, ABC with or without allograft resulted in no bone formation and instead formed a fibrous tissue which is dissolved in time [73].

Fig. 3 shows the posterolateral lumbar fusion by ABGS with synthetic ceramics as a CRM in rabbit model and allograft as CRM in sheep model. ABGS implants induced new bone formation which undergo remodeling and achieved a complete fusion of vertebrae between the two transverse processes. The newly induced bone trabeculi are fully integrated with trabeculi of transverse processes and the fusion is mechanically competent.

BMP6 is chosen in ABGS over BMP2 or BMP7 as 1) it reversibly binds with Noggin, a BMP antagonist abundant in bone [76] and thus help to lower the dose to effect osteogenesis, 2) utilizes most of BMP type I receptors (ALK2, ALK3 and ALK6) for signaling and 3) exhibits a high specific alkaline phosphatase activity in osteoblastic cell cultures [73]. Furthermore, it was shown that ABC reduced inflammatory and foreign body reactions when used with high mineral containing allograft or synthetic ceramics in PLF models [74,75,77]. These findings led to the evaluation of ABGS for safety and efficacy in a Phase I study in patients with Distal Radial Fractures [78] and a Phase I/II study in patients undergoing High Tibial Osteotomy [79].

5. Conclusion

BMPs serve as morphogenic signals for migration, proliferation and

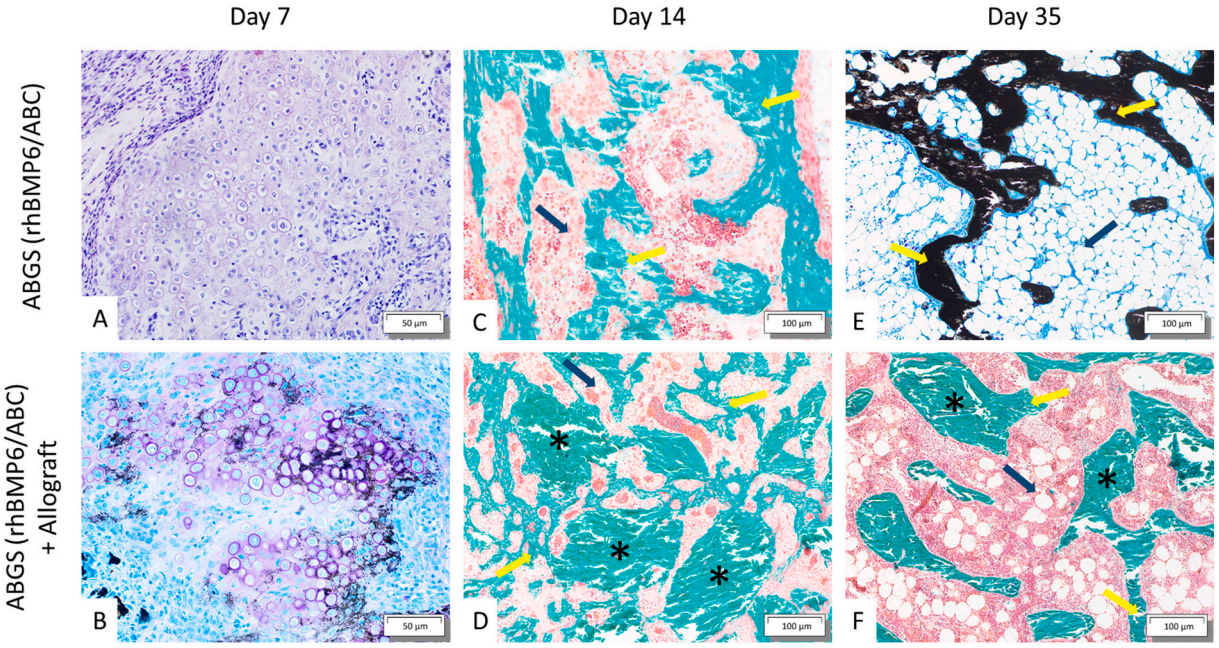

Fig. 2. Bone induction by ABGS without and with Allograft - rhBMP6 (20 µg) was formulated within 500 µL of autologous blood coagulum (ABC) and implanted in a rat subcutaneous site. A and B, represent rat implants harvested on day7; note extensive chondrogenesis and evidence of endochondral ossification. C and D, represent rat implants harvested on day 14; note extensive new bone formation (yellow arrows) and bone marrow differentiation (dark blue arrows). In implants containing allograft, newly formed bone is in close proximity to allograft particles (black asterisks) and a begining of creeping substitution is evident. E and F, represent rat implants harvested on day 35; note bone marrow (dark blue arrows) contains both hematopoetic and adipocytic components. Allograft particles are being resorbed by ongoing process of creeping substitution (yellow arrows). Modified from [73,74]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differentiation of mesenchymal stem cells for the formation of skeleton during embryogenesis. BMPs are potent inducers of new bone formation and shown to promote bone repair and regeneration in adults [54]. The outcome of tissue responsiveness for bone induction is dictated by the responding cells and by an appropriate carrier/scaffold under a permissive environment rather than by a BMP signal [80]. There are several osteogenic BMPs, BMP antagonists and BMP receptors expressed to govern bone formation during fracture repair and restoration. Extracellular matrices like heparan sulfate proteoglycans and type IV collagen that interact with BMP ligands add to that regulation. Thus far, two BMP based biologics, rhBMP2 and rhBMP7 containing bovine collagenous scaffold have been approved for clinical use for local bone formation [62,67]. However, there were numerous unwanted safety issues associated with bovine collagenous matrix as scaffold and high doses of BMPs employed in the current device [70]. The finding that autologous blood coagulum serves as a physiological carrier and in combination with rhBMP6 induces new bone formation, restores diaphyseal segmental defects [73] and promotes spinal fusion [74] at low doses suggested that some of these challenges could be avoided in the future.

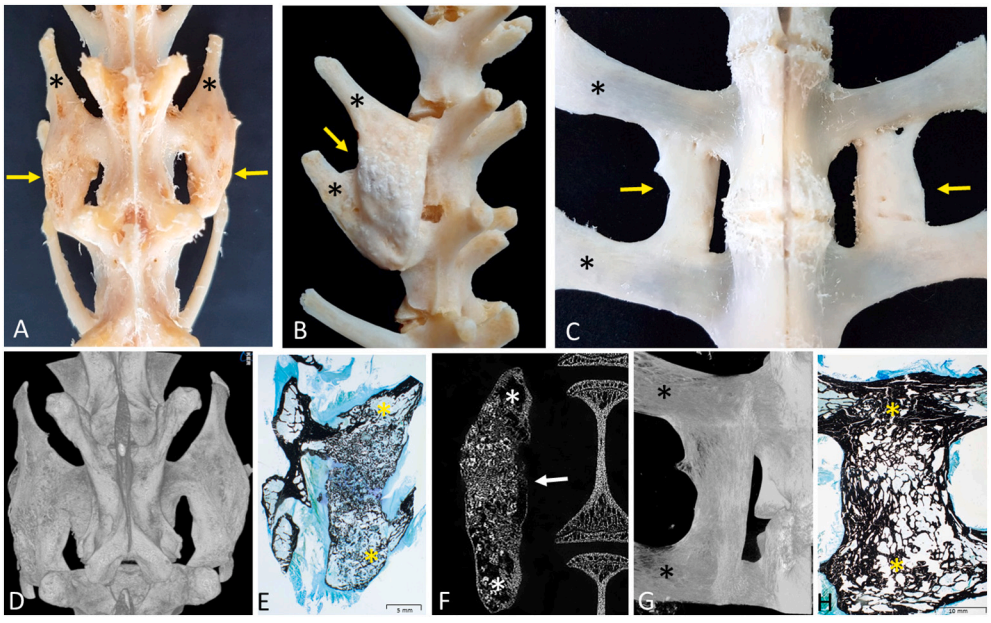

Fig. 3. Newly formed bone in rabbit and sheep model of posterolateral spinal fusion without instrumentation. A. Fused transverse processes in a rabbit treated with ABGS (2.5 mL ABC + 500 µg rhBMP) (modified from [74]); B. Newly formed bone in a rabbit treated with ABGS and synthetic ceramics (modified from [77]) C. Fused transverse processes in a sheep treated with ABGS (8 mL ABC + 1500 µg rhBMP and 2.4 g allograft) (modified from [75]); D. Micro CT reconstruction of A.; E. Undecalcified histology section stained with von Kossa of A; F. Frontal micro CT image of B; G. Micro CT image of C; H. Undecalcified histology section stained with von Kossa of C. Asterisks indicate transverse processes. Arrows (yellow and white) indicate newly formed bone between adjacent transverse processes. Scale bar in E and H as indicated.

Declaration of competing interest

TKS is Co-Founder and CEO of perForm biologics Inc., and SV is Co-Founder of perForm Biologics and Coordinator of EU HORIZON2020 (consortium partners) grant OSTEOproSPINE funding clinical studies of the new drug for bone repair (No. 279239).

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