Melatonin Promotes Osteoblast Differentiation and Bone Formation*

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Prior studies have demonstrated that the pineal hormone, melatonin, can stimulate chloramphenicol acetyltransferase activity in Drosophila SL-3 cells transfected with a chloramphenicol acetyltransferase reporter construct containing the response element of rat bone sialoprotein (BSP). Based on these findings, studies were performed to determine whether melatonin could similarly modulate the expression of BSP in two cell lines, the MC3T3-E1 (MC3T3) pre-osteoblast and rat osteoblast-like osteosarcoma 17/2.8 cell. Initial studies demonstrated that MC3T3 cells grown in the presence of 50 nM melatonin underwent cell differentiation and mineralization by day 12 instead of the 21-day period normally required for cells grown in untreated media. Melatonin increased gene expression of BSP and the other bone marker proteins, including alkaline phosphatase (ALP); osteopontin; secreted protein, acidic and rich in cysteine; and osteocalcin in MC3T3 cells in a concentration-dependent manner. Levels of melatonin as low as 10 nM were capable of stimulating transcription of these genes when cells were grown in the presence of β-glycerophosphate and ascorbic acid. Under these conditions, melatonin induced gene expression of the bone marker proteins; however, this does not occur until the 5th day after seeding the culture dishes. Thereafter, MC3T3 cells responded to melatonin within 2 h of treatment. The fully differentiated rat osteoblast-like osteosarcoma 17/2.8 cells responded rapidly to melatonin and displayed an increase in the expression of BSP, ALP, and osteocalcin genes within 1 h of exposure to the hormone. To determine whether melatonin-induced osteoblast differentiation and bone formation are mediated via the transmembrane receptor, MC3T3 cells were treated in the presence and absence of melatonin with either luzindole, a competitive inhibitor of the binding of melatonin to the transmembrane receptors, or pertussis toxin, an uncoupler of G, from adenylate cyclase. Both luzindole and pertussis toxin were shown to reduce melatonin-induced expression of BSP and ALP. These results demonstrate, for the first time, that the pineal hormone, melatonin, is capable of promoting osteoblast differentiation and mineralization of matrix in culture and suggest that this hormone may play an essential role in regulating bone growth.

Melatonin is the major hormone released from the pineal gland, and its levels are synchronized by environmental light with nightly plasma concentrations reaching approximately 50 times higher than that reached during daytime (1–3). Melatonin regulates a variety of physiological and pathophysiological processes including hypothalamic control of circadian rhythms (4–6), regulation of reproductive function in seasonally breeding species (7), and regulation of temperature (8), sexual development (9, 10), the immune system (11), and the cardiovascular (12). It has also been shown to influence cell differentiation where it can either stimulate or suppress cell division depending on its concentration or the cell type examined (13–15). In light of this, melatonin has been proclaimed to be a cure-all for everything from treating insomnia and cancer to acting as an anti-aging agent (16–18). In regard to melatonin actions as an anti-aging agent, it is known that secretion of the hormone is decreased during the aging process, and when administered to aging animals, it is capable of increasing their life span by almost 20% (16–18). Although little is known about the biological consequences of diminished melatonin production during aging, it is likely to have a profound effect on a variety of systems in vivo.

The recent isolation of transmembrane (19–21) and nuclear receptors (22–24) for melatonin has enabled scientists, for the first time, to characterize the biochemical and molecular mechanisms by which it regulates cell function. Three transmembrane receptors, Mel R1a, -1h, and -1c, have been identified and all are coupled to G, and capable of inhibiting cAMP formation (21, 22). Two putative nuclear receptors, RZRα1 and -β, have been identified and presumed to be responsible for the transcriptional control of a number of genes (23–25). These receptors share considerable sequence homology with the retinoic acid orphan receptor, RXR, and bind selectively as monomers to the response element (RGGTCA; R = A or G) found in several genes including that for bone sialoprotein (BSP), cellular retinoic acid-binding protein-I (CRBP-I), 5-lipoxygenase, and the inhibitor of cyclin-dependent kinases, p21WAF1/CIP1 (26–28). Whether the orphan receptor, RZR, represents the “true” binding protein for melatonin is not known, yet several studies have linked the actions of this hormone to this nuclear transcription factor. In this regard melatonin was reported to increase chloramphenicol acetyltransferase activity in Drosophila SL-3 cells transfected with the chloramphenicol acetyltransferase reporter gene containing the RZR/β response elements (28). In addition, melatonin was reported to down-regulate the expression of 5-lipoxygenase about 5-fold.

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1 The abbreviations used are: RZR, retinoid Z receptor; BSP, bone sialoprotein; ALP, alkaline phosphatase; OC, osteocalcin; OPN, osteopontin; AA, ascorbic acid; GP, β-glycerophosphate; SPARC, secreted protein, acidic and rich in cysteine; kb, kilobase pair(s); OP-1, osteogenic protein-1; CRBP-I, cellular retinoic acid-binding protein-I; FBS, fetal bovine serum; ROS, rat osteosarcoma cells.
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exclusively in cells that contain RZRs (27) and increase mRNA levels approximately 3-fold in the hippocampus of pinealectomized rats as compared with the sham operated controls (29).

Based on the above findings, studies were performed to determine whether melatonin could similarly modulate BSP expression in two cell lines, the MC3T3 pre-osteoblast (30) and ROS (31) osteoblast-like cell lines, both of which selectively express BSP during cell differentiation. BSP is an acidic, tyrosine-sulfated, extracellular matrix protein (32-36) that is expressed during osteoblastic cell differentiation and is required for mineralization (37-39). The presence of the response element for the RZR in BSP is interesting in light of the fact that melatonin has never been implicated in the regulation of bone disposition. The results of these studies reveal that melatonin can increase the expression of BSP as well as several other essential bone marker proteins including ALP and OC and, in addition, stimulate both osteoblast differentiation and mineralization.

MATERIALS AND METHODS

Cell Culture Conditions and Differentiation Assay—MC3T3 cells (a cloned osteoblastic cell line originated from newborn mouse calvaria) were plated (8 x 10^5 cells/60-mm dish) and grown to confluency in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and antibiotics (growth media). Media were changed every other day.

To study the spontaneous differentiation of MC3T3 cells into osteoblasts, cells were cultured for up to 21 days in growth media containing ascorbic acid (AA; 50 μg/ml) and β-glycerophosphate (GP; 10 mM) (differentiation medium). Melatonin, dissolved in Me2SO, was added to the culture media as described under “Results.” Control cultures received an equivalent quantity of Me2SO.

ROS cells (a gift from Dr. G. Rodan, Merck Sharp and Dohme Research Laboratories, West Point, PA) are an osteoblast-like cell line, were plated (7 x 10^5 cells/60-mm dish) and grown in Ham’s F-12 medium supplemented with 20 mM HEPES, 2.5 mM l-glutamine, 1.1 mM CaCl2, 5% FBS, and antibiotics (growth media). For time course studies, cells grown in 60-mm dishes were kept overnight in low serum (1%) containing growth media and then treated for 1-72 h with GP/AA and 50 nM melatonin.

Cell Proliferation Assay—MC3T3 cells and ROS cells were plated in 24-multiwell dishes (Becton Dickinson Laboratory, Lincoln Park, NJ) at a density of 5-10 x 10^4 cells/well and cultured for 24 h in growth media containing 10% FBS. To assess the effects of melatonin on cell proliferation, cells were incubated in growth media containing 2% FBS for 24 h and then treated for 1, 2, 4, and 6 days with the following: 1) GP and AA; 2) melatonin alone; 3) GP, AA, and melatonin; 4) GP, AA, and osteogenic protein-1 (OP-1, 40 ng/ml) (differentiation medium); and 5) no treatment. Cell number was assessed using a Coulter Z1 cell counter (Coulter Corporation, Miami, FL) after trypsinization and presented as the mean ± S.D. of triplicates.

Effects of Luzindole and Pertussis Toxin on Expression of ALP and BSP mRNA—Confluent MC3T3 cells grown on 60-mm dishes were treated with GP/AA and 50 nM melatonin in the presence and absence of either pertussis toxin (100 ng/ml) (List Biological Laboratories, Inc., Campbell, CA) or luzindole (0.2 and 2 μM) (Sigma) for the time indicated. Total RNA was isolated and subjected to Northern blot analysis as described below. In other experiments, cells cultured for 7 days in the presence of GP/AA were treated for 2 h with either 2 μM luzindole or 100 ng/ml of pertussis toxin. Cells were subsequently treated for an additional 2 h with 50 nM melatonin, and the RNA was isolated and subjected to Northern blot analysis.

Total RNA Isolation and Northern Blot Analysis—Total RNA was prepared by guanidinium isothiocyanate extraction using TRIzol reagent (Life Technologies, Inc.). RNA samples (10–20 μg/lane) were electrophoresed on a 1% agarose gel containing formaldehyde, transferred to to Kodak X-OMAT film at −80 °C using two intensifying screens. For

RESULTS

MC3T3 cells are pre-osteoblastic cells that differentiate into mature osteoblasts when grown in the presence of GP and AA (41). Differentiation of these cells to mature osteoblasts normally requires incubation in the presence of both GP and AA for at least 21 days at which time mineralization occurs (42). As illustrated in Fig. 1, melatonin (50 nM) induced cell differentiation by day 12 equivalent to or greater than that for cells grown for 21 days in the absence of the hormone. Along with increased differentiation, the time period for mineralization was also greatly accelerated in cultures treated with melatonin (Fig. 2). Cultures containing melatonin at day 12 showed extensive mineralization as compared with control cultures.

FIG. 1. Phase contrast micrographs of MC3T3 control and melatonin-treated cells. Cells were plated at a density of 8 x 10^4/mm-disc and grown to confluence (a), 12 days (b), and 21 days (c) after treatment with GP/AA only and 12 days after treatment with melatonin/AA (d). Cultures were photographed with an Olympus inverted microscope using phase contrast mode. Note the presence of an increased number of mineralized nodules (arrowsheads) in d.
grown for 21 days in the absence of melatonin.

As illustrated by the photomicrographs in Fig. 3, two weeks after treatment with melatonin, nodules were composed of multilayers of cells and densely packed collagen fibrils in the intercellular spaces with heavy mineral deposits preferentially located in the lower half of the nodules. As noted in the inset of Fig. 3, hydroxyapatite crystals were associated with the collagen fibrils.

Based on prior observations demonstrating that morphogenic agents such as OP-1, which stimulate osteoblast differentiation and mineralization, also suppress cell division (30), studies were performed to examine the effect of melatonin on MC3T3 and ROS cell proliferation. As indicated by the data in Fig. 4A, suppression of MC3T3 cell division by melatonin was equivalent to that produced by OP-1 at day 1 after initiating treatment. However, unlike OP-1, this inhibitory effect subsided by day 2 for cells grown in the presence of melatonin. As illustrated in Fig. 4B, melatonin also appeared to suppress cell proliferation in the fully differentiated ROS cells, although this response never reached statistical significance. Thus, unlike OP-1, melatonin (50 nM) may not fully suppress cell proliferation but instead may only delay entry of the osteoblast cells into the cell cycle after plating.

During MC3T3 cell differentiation induced by GP/AA, there is a selective and time-dependent induction of several proteins required for bone matrix formation. The mRNA for type I collagen, SPARC, and OP are all expressed early, and this is normally followed by increased mRNA expression of osteoblast markers such as BSP, ALP, and lastly OC. Because the gene for BSP has been reported to possess the response element for RZR, experiments were performed to determine the effect of melatonin on gene expression of BSP as well as the other proteins expressed during differentiation of MC3T3 cells. As illustrated in Fig. 5, concentrations of melatonin as low as 10 nM were sufficient to stimulate transcription of the BSP gene by day 12 after initiating treatment. Of the other marker proteins examined, expression of the ALP gene was also found to be up-regulated at similar concentrations of melatonin, suggesting that this gene may also be directly responsive to the actions of the purported nuclear receptor for melatonin, RZR.

In these experiments, melatonin had considerably less effect on the gene expression of OPN, SPARC, and collagen as compared with the control cultures grown in differentiation media. There was a very small increase in OC mRNA levels in cultures treated with 50 nM melatonin, but this could only be detected by exposing the blots to the film for 5-7 days. These data demonstrate that melatonin is capable of consistently stimulating the expression of the major proteins during MC3T3 cell differentiation.

It was also of interest to determine the time course during MC3T3 cell differentiation induced by melatonin that increased gene expression of BSP and ALP occurred. As illustrated by the data in Fig. 6, the observed increase in expression of the BSP and ALP genes was not detected until sometime between days 5 and 9 after continuous exposure to the hormone. Increased expression of ALP occurred by day 5 and developed prior to that of BSP, consistent with previous studies demonstrating that ALP expression occurs early during osteoblast differentiation. Along with increased expression of BSP and ALP, we also observed increases in mRNA levels for OPN and SPARC when compared with that in control cultures. Like ALP, increased expression of OPN is also an early event marker for osteoblast differentiation, and this increase is observed by day 5 after beginning treatment with melatonin. In contrast, the appearance of SPARC during MC3T3 differentiation resembled that for BSP. Melatonin again had little effect on either type I collagen or OC expression, although, as noted above, there was a small but significant increase in the expression of the latter gene by day 12 when membranes were exposed to film for 7 days. Control cultures maintained in the absence of melatonin required longer incubation times for gene expression of the bone marker proteins.

The delay observed in melatonin-induced expression of mRNA for BSP and ALP suggests that during the early phases of cell differentiation either de novo synthesis or activation of one or more essential proteins must occur in order for the cells to respond to melatonin. Thus, it was of interest to determine the time point during differentiation when MC3T3 cells first become responsive to melatonin. For these experiments, cells were grown in differentiation media in the absence of melatonin for the time periods specified in Fig. 7. Melatonin was added at the specified time periods, and 2 h later cells were screened for expression of the bone marker proteins. Results of these experiments demonstrated that MC3T3 cells must undergo differentiation for a minimum of 5-7 days before melatonin is capable of stimulating the expression of BSP and ALP.

In these experiments, melatonin was also found to increase the expression of both collagen and OC genes.
Based on these results, it may be expected that melatonin would induce gene expression of the bone marker proteins more rapidly in a fully differentiated osteoblastic cell that possibly already expresses the necessary components to respond to melatonin. The ROS cell is a fully differentiated osteoblast-like cell line that expresses bone marker proteins when grown in the presence of GP/AA. As illustrated in Fig. 8, when these cells were exposed to 50 nM melatonin in the presence of GP/AA, there was a rapid increase in the expression of BSP and OC genes. Induction of these three genes in ROS cells was observed within 1 h of treatment with melatonin and was not effected by cycloheximide treatment for 4 h (data not shown), indicating that de novo synthesis of additional proteins is not required for increased expression of the three bone marker protein genes.

To determine whether melatonin-induced osteoblast differentiation and bone formation are mediated via the interaction of the hormone with the transmembrane receptor, MC3T3 cells were treated in the presence and absence of melatonin with either luzindole, a competitive inhibitor of the binding of melatonin to the transmembrane receptors (20), or pertussis toxin, an uncoupler of G, from adenylate cyclase (22). Expression of mRNA for ALP and BSP was examined using Northern blot analysis. Results of these studies, illustrated in Figs. 9 and 10, reveal that both luzindole and pertussis toxin are capable of reducing melatonin-induced expression of both BSP and ALP. As illustrated in Fig. 9, concentrations of luzindole as low as $2 \times 10^{-7} \text{M}$ were found to completely block melatonin-induced increases in the expression of both BSP and AP at 9 and 12 days of treatment as compared with cells treated with melatonin alone. Similar results were observed (Fig. 10) after treatment with pertussis toxin, suggesting that melatonin-induced expression of both BSP and ALP is mediated by a cAMP-dependent process initiated by the binding of the hormone to its transmembrane receptor.

To demonstrate that the effects of luzindole and pertussis toxin were not caused by their excessive accumulation in the above studies, experiments were performed in which cells were exposed to these agents for 2 h in the presence of melatonin. In these experiments, MC3T3 cells were initially pretreated with either luzindole or pertussis toxin for 2 h prior to the addition of melatonin. Cells were incubated for an additional 2 h, and mRNA levels for BSP and ALP were subsequently assessed. As illustrated in Fig. 11, luzindole suppressed melatonin-induced expression of BSP, whereas pertussis toxin suppressed expression of both BSP and ALP. These data are consistent with the effects of melatonin being mediated by the transmembrane receptor.

**DISCUSSION**

MC3T3 and ROS cells were chosen for this study because both cell lines produce BSP and undergo cell differentiation and mineralization when exposed to a variety of agents that

**Fig. 4. Effect of melatonin and OP-1 on MC3T3 and ROS cell proliferation.** MC3T3 (A) and ROS (B) cells were plated in 24-multiwell plates at a density of $2 \times 10^4$ cells/well and cultured for 24 h in growth media containing 10% FBS. To assess the effects of melatonin and OP-1 on cell proliferation, cells were incubated in growth media containing 2% FBS for 24 h and then treated for 1, 2, 4, and 6 days with GP/AA, GP/AA/melatonin (GA/Me), melatonin alone, GP/AA/OP-1 (GA/OP-1), or no treatment (No Tx.). Cell number was quantitated using a Coulter Z1 cell counter and presented as the mean $\pm$ S.D. of triplicates.

**Fig. 5. Dose responses of melatonin on the expression of mRNAs for ALP and bone matrix components.** Confluent MC3T3 cells were grown for 0 and 12 days in differentiation media containing various concentrations of melatonin as indicated. Total RNA was isolated, and mRNA for bone matrix components was assessed by Northern blot analysis using $^{32}$P-labeled cDNA probes. In the specific case of OC, x-ray films were developed after exposure for 2 (OC-2) and 7 (OC-7) days.
increase the expression of this gene. Based on studies in the literature revealing that BSP contains the appropriate response element for RZR, studies were undertaken to determine whether melatonin could also stimulate the expression of this regulatory protein and induce differentiation and matrix mineralization in these osteoblastic cell lines. As one of the major secretory proteins of osteoblasts, BSP functions to regulate mineralization possibly by its direct interaction with cell surface integrin receptors (37, 38) and/or by initiating nucleation of the bone mineral, hydroxyapatite (39). Increased expression of BSP along with that of several other bone marker proteins is required to induce mineralization. When MC3T3 cells are grown in the presence of GP/AA, BSP expression and mineralization are normally not observed until 21 days after initiating differentiation media containing melatonin/GP/AA for the time specified. Total RNA was isolated, and mRNA for bone matrix components was assessed by Northern blot analysis using 32P-labeled cDNA probes. In the specific case of OC, x-ray films were developed after exposure for 2 (OC-2) and 7 (OC-7) days.

Fig. 6. Northern blot analysis of mRNAs for ALP and bone matrix components in MC3T3 cells cultured in the presence of GP/AA or melatonin/GP/AA. Confluent MC3T3 cells were grown in differentiation media containing melatonin/GP/AA for the time specified. Total RNA was isolated, and mRNA for bone matrix components was assessed by Northern blot analysis using 32P-labeled cDNA probes. In the specific case of OC, x-ray films were developed after exposure for 2 (OC-2) and 7 (OC-7) days.

Fig. 7. Northern blot analysis of the expression of mRNAs for ALP and bone matrix components after a 2-h melatonin treatment of MC3T3 cells. Confluent MC3T3 cells were cultured in the presence of GP/AA for 0, 5, 7, and 9 days and then treated for 2 h with melatonin (50 nM) prior to isolation of RNA. Total RNA was isolated, and mRNA for bone matrix components was assessed by Northern blot analysis using 32P-labeled cDNA probes. In the specific case of OC, x-ray films were developed after exposure for 2 (OC-2) and 7 (OC-7) days.

Fig. 8. Time course for the expression of mRNAs for ALP and bone matrix components in ROS cells. Confluent cells grown on 60-mm dishes were kept overnight in growth media containing 1% fetal calf serum. Cells were subsequently treated for 1–72 h in the presence of GP/AA, or melatonin/GP/AA, as indicated. Total RNA was isolated, and the expression of mRNA for the different bone matrix components was assessed by Northern blot analysis using 32P-labeled cDNA probes.

Fig. 9. Effects of luzindole on MC3T3 cell differentiation. MC3T3 cells were treated with GP, AA, and 50 nM melatonin or GP, AA, melatonin, and luzindole, a competitive inhibitor of the binding of melatonin to the transmembrane receptor. Total RNA was isolated, and expression of ALP and BSP was examined using Northern blot analysis on the days indicated.

Fig. 10. Effects of pertussis toxin on MC3T3 cell differentiation. MC3T3 cells were treated with GP, AA, and 50 nM melatonin or GP, AA, melatonin, and pertussis toxin (100 ng/ml), an inhibitor of Gi and cAMP formation. Total RNA was isolated, and expression of ALP and BSP was examined using Northern blot analysis on the days indicated.
treatment (41). A number of hormones and growth factors can stimulate the expression of BSP as well as ALP, SPARC, OPN, and OC and thus, facilitate MC3T3 cell differentiation and mineralization (43, 44, 31, 46). These include glucocorticoids (43), OP-1 (46), parathyroid hormone (44), and transforming growth factor-β (31). Results reported in this paper reveal that melatonin can similarly promote MC3T3 cell differentiation and mineralization. However, unlike these other growth factors, melatonin only slightly suppresses MC3T3 cell growth and only minimally increases the expression of OC.

In an attempt to ascertain whether the transmembrane receptors are involved in mediating the actions of melatonin, studies were performed to determine whether short and long term exposure to luzindole and pertussis toxin could attenuate the actions of melatonin. Both long and short term exposure to luzindole and pertussis toxin diminished ALP expression upon short term exposure. These data suggest that the transmembrane receptor via signaling through the G protein is likely initiating the actions of melatonin in MC3T3 cells. This is further supported by our preliminary findings tentatively identifying the Mel R1b receptor in these cells. The question still remains as to whether the putative nuclear orphan receptor, RZR, is also involved in mediating the actions of this hormone in MC3T3 cells. Because RZR is reported to be regulated by cAMP (47), it is reasonable to assume that the binding of melatonin to its transmembrane receptor will result in changes in the levels or activity of this nuclear transcription factor leading to alteration in the expression of BSP and ultimately osteoblast differentiation.

Further evidence linking RZR with the observed melatonin-induced morphological changes in MC3T3 cells stems from the findings that the expression of ALP, OPN, SPARC, and OC along with BSP are all induced by melatonin. In this regard, it should be noted that the genes for these proteins also contain the appropriate base sequence, RGGTCA, within their promoter region required for RZR binding (48–52). In addition, preliminary studies with MC3T3 cells have further demonstrated that melatonin can suppress the expression of CRBP-I, a regulatory protein found in osteoblasts (53) that also contains the RZR response element (45). Interestingly, the concentrations of melatonin found to suppress CRBP-I in MC3T3 are identical to that required to stimulate BSP expression, suggesting a common signaling mechanism for the two. The observation that melatonin can down-regulate the expression of CRBP-I is similar to the response observed with melatonin in human B lymphocytes for 5-lipoxygenase (27). These studies suggest that RZR, at the minimum, may lie in the pathway by which melatonin regulates transcription of the genes for BSP, CRBP-I, and possibly the other bone marker proteins as well.

Our results reveal that MC3T3 cells must first undergo cell differentiation in the presence of GP/AA for approximately 5 days before they become responsive to melatonin. At this time, melatonin is capable of stimulating the gene expression of bone marker proteins within 2 h. A similar rapid response time to melatonin is also found in the fully differentiated ROS cells. This suggests that ROS cells, which are already fully differentiated, do not require the de novo protein synthesis of a regulatory factor responsible for controlling the actions of melatonin. In addition, preliminary studies have demonstrated that melatonin can also stimulate differentiation of primary osteoblast cells grown in culture, confirming that the actions of melatonin are not restricted to transformed cells.

In summary, the results reported in this paper demonstrate that the pineal hormone, melatonin, is capable of promoting differentiation and mineralization of osteoblast cells grown in culture. This is the first demonstration that melatonin has the potential to play an essential role in regulating bone growth. These findings place melatonin with a select handful of other agents including glucocorticoids, bone morphogenic proteins, and vitamin D₃ that are known to stimulate mineralization in osteoblasts (30, 42). Because melatonin levels decrease during the aging process, the possibility must be considered that it may have a significant influence on the rate of synthesis and/or maintenance of bone in the elderly. As to whether this decrease in melatonin during aging contributes to osteoporosis or whether treatment with melatonin can prevent this disorder is not known but is currently under investigation.

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