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Effects of GDF7/BMP12 on Proliferation and Alkaline Phosphatase Expression in Rat Osteoblastic Osteosarcoma ROS 17/2.8 Cells

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Abstract Growth and differentiation factor 7(GDF7), also later called as bone morphogenetic protein (BMP)12, is a new member of the BMP superfamily, which induces formation of tendon-like tissue formation in the ectopic implantation experiments. We examined the effect of BMP12 on proliferation and expression of phenotype-related genes in rat osteoblastic osteosarcoma ROS17/2.8 cells. BMP12 treatment enhanced proliferation of ROS17/2.8 cells within 3 days and this effect was observed at least up to day 6 of the treatment. The cell number was increased by about 50% on day 3 and about two-fold by day 6. These effects were observed at the dose range between 40 and 1,000 ng/ml. Treatment with BMP12 also enhanced alkaline phosphatase activity by about 50% in ROS17/2.8 cells within 24 h of the treatment. The effect peaked at 48 h and was still observed at 72 h. The enhancing effect of BMP12 on alkaline phosphatase was observed similarly at the doses ranging from 40 to 1,000 ng/ml. These data indicate that BMP12 has positive effects on proliferation and phenotypic expression of ROS 17/2.8 cells. J. Cell. Biochem. 72:177–180, 1999.

Key words: GDF7; osteoblast; growth factor; proliferation; differentiation

Bone morphogenetic proteins (BMPs) are the members of TGFβ superfamily and are playing crucial roles not only in osteogenesis but also in formation or induction of various tissues and organs during development. BMPs form a distinct group consisting of four subfamilies which are classified according to the homology in their mature regions [Hogan, 1996]. One of these subfamilies consists of growth/differentiation factor (GDF) 5 (cartilage derived bone morphogenetic protein [CDMP]-1) [Storm et al., 1994; Chang et al., 1994], GDF7 (BMP12) [Storm et al., 1994; Celeste et al., 1995], and BMP13 (GDF6) [Storm et al., 1994; Dube and Celeste, 1995]. These three GDFs were identified recently by degenerative PCR and their mature regions are close to one another except the glycine-rich insert in GDF7 [Storm et al., 1994].

Mutations in Gdf5 gene cause brachypodism (bp) in mice [Storm et al., 1994]. Bp mice are featured by their short limbs with joint anomalies, while the mice do not show major changes in axial skeletal structures [King et al., 1996]. Furthermore, cartilage- and bone-inducing activity of GDF5/CDMP-1 was demonstrated [Chang et al., 1994; Hötten et al., 1996]. Together with these observations, the expression pattern of their transcripts during development suggests that GDF5 regulates formation of bones and joints in limbs [Storm et al., 1994; Chang et al., 1994; Hötten et al., 1996; Storm and Kingsley, 1996]. More recently, BMP12 was identified to be the human homologue of murine GDF7, which was found prior to BMP12, based on the comparison of their predicted amino acid sequences [Celeste et al., 1995].

Ectopic implantation studies of BMP12 demonstrated that it induced formation of tendon/ligament-like tissues in subcutaneous tissues

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[Wolfman et al., 1995] or in muscle [Cox and Rosen, 1996]. Ultrastructural analysis and Northern analysis of the ectopically induced tendon/ligament-like tissues indicated that the tissue revealed several features characteristic to tendon and ligament tissues [Wolfman et al., 1995; Cox and Rosen, 1996]. Furthermore, it was reported that BMP12/GDF7 was expressed at the site of developing joints in mouse embryos [Wolfman et al., 1995]. Because tendons and ligaments attach to bones, formation of tendon and ligament is coordinated with that of bone. It was also indicated that BMP12 inhibited muscle differentiation and did not direct myoblast to become osteoblastic cells in vitro [Inada et al., 1996]. However, BMP12 effects on osteoblastic cells per se was not studied in detail. We report here that BMP12 stimulates both proliferation and expression of alkaline phosphatase in osteoblastic osteosarcoma ROS17/2.8 cells.

MATERIALS AND METHODS

Cell Culture

ROS17/2.8 or ROS25/1 cells were grown in modified Ham's F-12 nutrient mixture [Majeska et al., 1980] (Gibco BRL, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) on 10 cm diameter dish (Coster Corp. Cambridge, MA). For experiments, confluent cells were fed with fresh media 1 day before harvesting with 0.125% trypsin. The cells were plated into 2 cm^2 well (Nunc A/S Roskilde, Denmark) at 20,000 cells/cm^2 and cultured overnight. On the next day, the cells were rinsed twice with serum-free F-12 and were cultured in the presence or absence of BMP12 in serum-free medium. The day when BMP12 treatments started was defined as the day 0.

Cell Count

At several time points, the cells were harvested with 0.125% trypsin and were resuspended in 9 ml Isoton III(Coulter Electronics Ltd. Beds, England) in counting vials, and counted by Coulter Counter Model ZM (Coulter Electronics Ltd. Beds, England).

Alkaline Phosphatase Assay

The cells were lysed and scraped into 0.25 ml of a buffer containing 10 mM Tris HCl pH 7.5, 0.5 mM $MgCl_2$, 0.1% Triton X-100. These cell lysates were homogenized by freeze-and-thaw followed by sonication. The AP activity in the samples were assayed using Na^2 p-nitrophenyl phosphate(PNPP) as substrate. Protein contents in each sample were determined according to Coomassie blue G method [Noda et al., 1990].

Statistical Analysis

Statistical evaluation was conducted by employing Student's *t*-test.

RESULT

To investigate whether BMP12 affects proliferation of the cells in osteoblastic lineage, we first examined its effect on ROS 17/2.8 cells. Treatment with 400 ng/ml BMP12 increased the number of ROS17/2.8 cells within 3 days under serum-free condition while no increase was observed in the control group. The increase by the BMP12 treatment was observed up to day 6 in culture (Fig. 1). We then examined several doses of BMP12 with regard to the effect on cell growth. ROS17/2.8 cell were treated with BMP12 at 40, 400, and 1,000 ng/ml. After the treatment for 4 days, cell numbers in the groups treated with BMP12 at the above-mentioned dose range increased by 60–90% compared with that of control group (Fig. 2). These results indicate that BMP12 stimulates proliferation of osteoblastic ROS17/2.8 cells.

We next examined the effect of BMP12 on the expression of osteoblastic phenotype in ROS17/2.8 cells. We examined alkaline phosphatase (AP) activity as a phenotypic marker which is

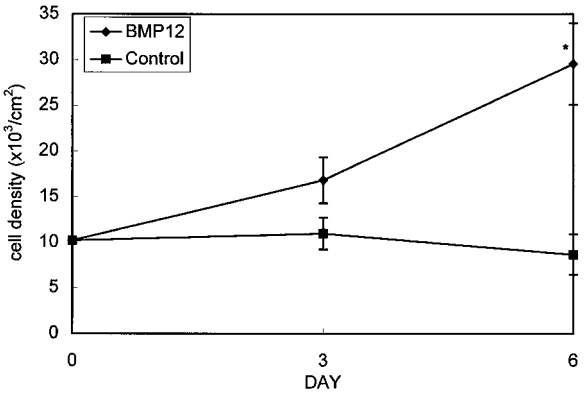

Fig. 1. Time-course of the BMP12 effect on cell growth of ROS 17/2.8 cells. The cells were treated with 400 ng/ml BMP12 (circle) or vehicle (square) as a control. After treatment for 3 and 6 days, these cells were harvested and counted. The media were replaced on day 3. The data represent mean ± S.E.M. of the cells in four wells obtained from one of two independent experiments with similar results. Asterisks indicate statistically significant difference against control. *$P < 0.05$.

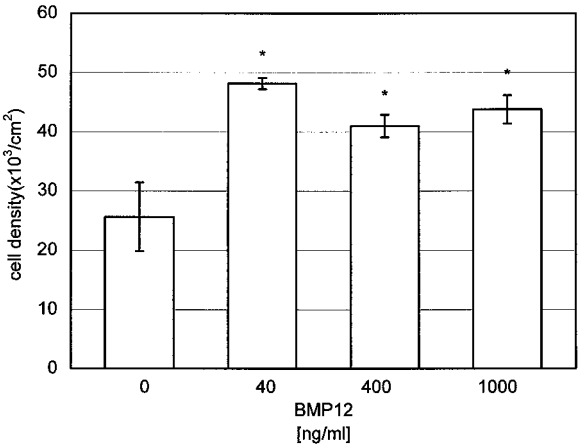

Fig. 2. BMP12 effects on the growth of ROS 17/2.8 cells. The cells were treated with 0, 40, 400, or 1,000 ng/ml BMP12 for 4 days. The cells were harvested and counted. The media were changed on day 3. The data represent mean ± S.E.M. of four samples for each treatment. Asterisks indicate statistically significant difference against control. *$P < 0.05$.

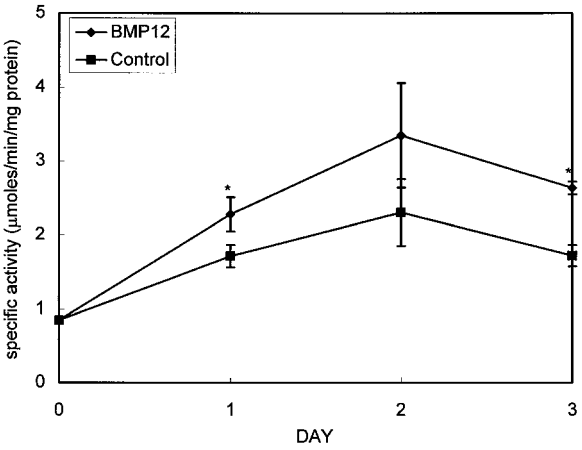

Fig. 3. Time-course of BMP12 effect on alkaline phosphatase activity in ROS 17/2.8 cells. The cells were treated with 400 ng/ml BMP12 (circle) or vehicle (square). After treatment for 1, 2, and 3 days, the cells were harvested and were assayed as described in Materials and Methods section. The data represent mean ± S.E.M. of eight (day 0), four (days 2 and 3) samples. Asterisks indicate statistically significant difference against control. *$P < 0.05$.

expressed during differentiation of osteoblastic cells although it is not absolutely specific to the cells in osteoblastic lineage. We treated ROS17/2.8 cells with 400 ng/ml of BMP12 for 1, 2, and 3 days. For each time point, the AP specific activity of the groups treated with BMP12 was higher than that of the control group (Fig. 3). AP activity was similarly enhanced by BMP12 treatment within a range of doses at 40 and 400 ng/ml in these cells (Fig. 4). The BMP12 effect

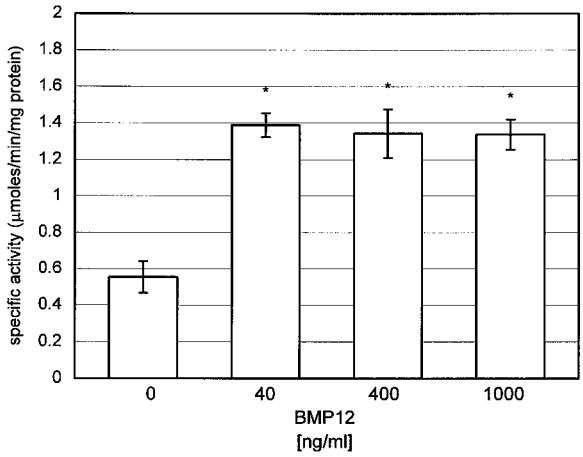

Fig. 4. BMP12 effect on alkaline phosphatase activity in ROS 17/2.8 cells. The cells were treated with 0, 4, 40, 400, and 1,000 ng/ml BMP12 for 3 days. The cells were harvested and assayed as described in Materials and Methods. The data represent mean ± S.E.M. of four samples for each treatment. Asterisks indicate statistically significant difference against the control. *$P < 0.05$.

on ROS17/2.8 cells was specific since BMP12 did not increase alkaline phosphatase activity and only slightly increased cell number in the less mature type cells, ROS25/1 [data not shown].

DISCUSSION

In this study, we report that BMP12 enhances proliferation and expression of alkaline phosphatase (AP) in ROS 17/2.8 cells. It was reported that BMP12 induced formation of tendon/ligament-like tissues when implanted ectopically [Wolfman et al., 1995; Cox and Rosen, 1996] and that BMP12 transcripts are expressed in joints of mouse embryos [Wolfman et al., 1995]. Our observation on BMP12 regulation of osteoblasts may suggest its possible roles in the sites common for both osteoblasts or tendon cells such as those in the insertions of the tendon-ligaments to bones. Alternatively, during the differentiation pathways starting from mesenchymal cells to osteoblasts and tendon/ligament cells, BMP12 sensitive fractions of the cells may represent a certain group of pluri-potential progenitors. In this regard, some fraction in the ROS-17/2.8 cells could be those which are relatively immature and could be corresponding to such pluri-potential cells, since we observed previously that these cells are heterogeneous with regard to the levels of alkaline phosphatase even though they are clonal cells [Noda M, unpublished data]. The relatively low levels of the BMP12 effects could be also due to

the relatively small size of the fraction of responding cells. To investigate the effect on phenotypic expression, we measured AP activity of these cells. Although it was reported that BMP12 did not increase AP activity in these cells in the presence of 5% serum [Inada et al., 1996], AP activity in BMP12 treated cells was enhanced compared to that in the control cells in serum-free condition in our experiments. This difference in the responses of the cells is possibly due to the treatment condition and may imply that the effect of BMP12 might be masked by the presence of serum which contains a number of factors. We compared the cell number as well as AP activities in ROS17/2.8 cells treated with three different doses of BMP12. BMP12 enhanced cell growth and AP activity similarly irrespective of the doses tested suggesting that the saturation dose for enhancement is also less than 40 ng/ml.

It is still not known which receptors expressed on ROS 17/2.8 cells could mediate the signal of BMP12. Recently, it was reported that GDF5, which is highly related to BMP12, preferentially bound to BMP receptor type I B(BMPRIB) [Nishitoh et al., 1996]. Since not many types of BMP receptors have been reported [Yamashita et al., 1996], it is likely that BMP12 may share one or several of the already identified BMP receptors, however we cannot exclude the possibility of the presence of unknown specific receptors which could mediate BMP12 signal. Presence or absence of serum may change the activity of BMP receptors via certain kinases, though this point also needs further investigation.

In conclusion, we showed that a new BMP family member, BMP12, which induces tendon/ligament like tissue enhanced proliferation and AP expression in ROS 17/2.8. The stimulatory effects of this protein on both growth of and phenotypic expression in ROS17/2.8 cells suggest that BMP12 could be involved in the regulation of the functions as one of the positive regulatory factors for osteoblasts.

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