Effect of water soluble extract of nacre (Pinctada maxima) on alkaline phosphatase activity and Bcl-2 expression in primary cultured osteoblasts from neonatal rat calvaria

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The nacre (mother of pearl) layer of the oyster Pinctada maxima shell can initiate bone formation by human osteoblasts *in vivo* and *in vitro* and is a new biomaterial that induces osteogenesis. This activity of nacre could be due to its water-soluble matrix. We examined the action of a water-soluble extract of nacre on the osteoblast phenotype of cells isolated from rat neonatal calvaria by measuring alkaline phosphatase (ALP) activity and by localization of the anti-apoptotic protein Bcl-2 by immunocytochemistry. ALP activity was increased 7% ($p < 0.001$) by 100 μg proteins/ml extract and 20% ($p < 0.001$) by 50 μg proteins/ml extract, but a low concentration of extract decreased the ALP activity by 8%. Cells treated with a high aspartic acid content fraction of the extract had increased ALP activity (23%, $p < 0.0001$). Nacre extract and the fraction have no effect on the proliferation of mature osteoblasts. Immunoreactive Bcl-2 was overproduced in the cytoplasm and nuclei of osteoblasts at all stages of culture. Bcl-2 was found over the whole chromatin in quiescent and mitotic cells at the end of mitosis in the two nuclei in one cell, before cytodieresis. Bcl-2 was also found over chromosomes. Thus, nacre extract stimulates Bcl-2 production in osteoblasts, that is correlated with the cell cycle. Bcl-2 was also abundant in the nucleoli of extract-treated cells. Thus, the concentration and subcellular distribution of Bcl-2 in osteoblasts in primary cultures is influenced by nacre extract, and related to the cell cycle and the regulation of gene expression. Hence, knowledge of how water-soluble extracts of Pinctada maxima nacre act on osteoblasts *in vitro* may reveal the mechanisms involved in its action *in vivo* on bone cells and bone regeneration.

1. Introduction

The nacre (mother of pearl) layer of the giant oyster (Pinctada maxima) shell can initiate bone formation *in vitro* [1] and *in vivo* [2]. Nacre is biocompatible and has osteogenic properties [3]. Nacre implants in sheep [4, 5], rats [6] and human for alveolar bone defects [2] caused differentiation of osteoblasts leading to the formation of mature, functional bone (see review in [7]). Nacre also initiates mineralization by human osteoblasts *in vitro* [8]. Lopez *et al*. have suggested that nacre acts on osteogenesis via its organic matrix, which contains diffusible, water-soluble factors [9]. The water-soluble matrix of the nacre from shells of other molluscs has been analyzed. The soluble matrix of Pinctada margaritifera var-cumigi nacre has a high concentration of carboxylic amino acids (aspartic and glutamic acids) [10], while that of the pearl oyster Pinctada fucata contains proteins with two functional domains, an acidic Gly-Asp, or Glu domain, and a carbonic anhydrase domain [11]. The soluble polyanionic proteins of the organic matrix of mollusc shells are templates for shell formation [12].

This study investigates the effects of a water-soluble extract of Pinctada maxima nacre on osteoblasts isolated from neonatal rats calvariae in primary culture. Osteoblasts from fetal rat calvaria can grow in culture and develop into mature osteoblasts that form mineralized nodules [13]. This development follows a temporal sequence of expression of the genes encoding growth-related proteins and osteoblast phenotypic markers [14]. Alkaline phosphatase (ALP) is present early in osteoblast development and is an indicator of

osteoblast differentiation (reviewed in [15, 16]). High ALP activity is generally considered to be a major marker of the osteoblast phenotype. Other cell types, like fibroblasts, have low ALP activity. We therefore measured the ALP activity of osteoblasts in control and nacre water-soluble extract cultures. We checked the effect of the extract by using primary cultures of osteoblasts isolated by sequential enzymatic digestion [17] from parietal bones of neonatal rats. These cells have the characteristics of osteoblasts: high alkaline phosphatase activity, high type I collagen synthesis, cyclic adenosine monophosphate (CAMP) and intracellular calcium responses to parathyroid hormone, and an osteocalcin response to 1,25-dihydroxy-vitamin D_3 [18]. Alkaline phosphatase activity and the synthesis of an anti-apoptotic protein, encoded by the proto-oncogene Bcl-2 are both characteristic of bone cells, osteoblasts and osteocytes [19] *in vivo*, and in cultures of osteoblasts [20]. These features were used to test the effects of a short incubation (48 h) with water-soluble nacre extract on the development of the osteoblast phenotype *in vitro*.

2. Materials and methods

2.1. Water-soluble nacre extracts

Dialyzed and lyophilized water-soluble extract obtained according to the procedure described in [9] and a fraction isolated by high performance liquid chromatography (HPLC) which has a high aspartic acid content were used for ALP activity measurement. The water-soluble extract was used for immunocytochemical analysis. The water-soluble extract was added to the cultures at concentrations of 100, 50, 25 and 1 µg protein/ml. Protein concentration was determined using the BCA Protein Assay (Pierce).

2.2. Isolation and cell culture

Two-day-old Wistar rats were from the INRA breeding facility at Jouy-en-Josas (France). Osteoblasts were isolated from the parietal bones by sequential enzymatic digestion [17]. They were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum in 96-well plates for alkaline phosphatase measurement and in the same conditions in 12-well plates on glass slides for immunocytochemical analysis.

2.3. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured *in situ* in microtiter plates. Cells were seeded in 96-well plates at 3,000 cells/well. Confluent cells were then incubated with nacre extract for 48 h. The medium was then removed, the cells washed twice *in situ* with 0.2 ml Tris-HCl buffer (50 mM Tris, 150 mM NaCl, pH 7.6) and ALP substrate (para-nitrophenylphosphate, 1.33 mg/ml, 0.2 ml/well) in TBS was added. The plates were incubated at 37 °C for 30 min. The hydrolyzed p-nitrophenylphosphate was measured in a plate reader at 405 nm. ALP specific activity is expressed as nmol para-nitrophenol liberated/min/cell. Statistical analysis were performed using Student's *t* test.

2.4. Quantitative cell staining

The osteoblasts in the wells of the lower half of the plate were stained with methylene blue [21] and counted to determine the total number of cells in each well. Dye was concentrated within the nuclei. Cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with water and air-dried overnight. The dried cells were rinsed with 0.1 M boric acid (pH 8.5) and incubated in 0.1 ml borate buffer containing 1% methylene blue for 60 min at room temperature, rinsed with water and air-dried overnight. The methylene blue was eluted from the cells with 0.2 ml 0.1N HCl (60 min at 37 °C). The OD of the eluted methylene blue was measured at 620 nm in a plate reader and 1.0 OD unit was assumed to be equivalent to 10^4 cells [21].

2.5. Immunocytochemical detection of Bcl-2

Proliferating osteoblasts (3 days in culture), subconfluent osteoblasts (6 days in culture) and confluent osteoblasts (9 days in culture) were fixed in acetone at − 20 °C for 5 min, rinsed in phosphate-buffered saline (PBS) pH 7.4 and treated with 10% methanol in H_2O_2 10% for 10 min. They were then incubated with rabbit anti-Bcl-2 polyclonal antibody (Santa-Cruz, CA, USA), diluted 1/400–1/200 in PBS-BSA (bovine serum albumin), overnight at 4 °C, washed with PBS/BSA, incubated with biotinylated anti-rabbit IgG (1/100) for 2 h at room temperature, rinsed and treated with streptavidin-horseradish peroxidase conjugate (Amersham) diluted 1/300 for 1 h at room temperature. Lastly, the cells were washed in PBS-BSA and incubated with diaminobenzidine (DAB, 10 mg in 15 ml 0.035 Tris-HCl with 15 µl H_2O_2, pH 7.66) for 30–60 min at room temperature, rinsed in Tris-HCl and mounted in buffered glycerine for examination under the light microscope (DMRB, Leica). The immunoreactivity was checked by incubating control cultures with non-immune serum.

3. Results

3.1. Effects of nacre extracts or a fraction with high aspartic acid content on ALP activity and the proliferation of rat osteoblasts

The effect of nacre extract was dose-dependent (Fig. 1A). Low doses of extract (1 µg/ml and 10 µg/ml) had no effect on ALP activity. 25 µg/ml caused a significant decrease (8%, $n = 120$, $p < 0.05$), whereas 50 µg/ml (20%, $n = 120$, $p < 0.0001$) and 100 µg/ml (7%, $n = 120$, $p < 0.001$) caused significant increases. Cell proliferation was unaffected, whatever the concentration of water soluble extract (Fig. 1B). Fraction with high aspartic acid content (enriched extract) (50 µg/ml) significantly increased ALP activity (23%, $n = 40$, $p < 0.0001$), whereas 100 µg/ml was without effect (Fig. 2A). This fraction had no effect on cell proliferation at any concentration (Fig. 2B).

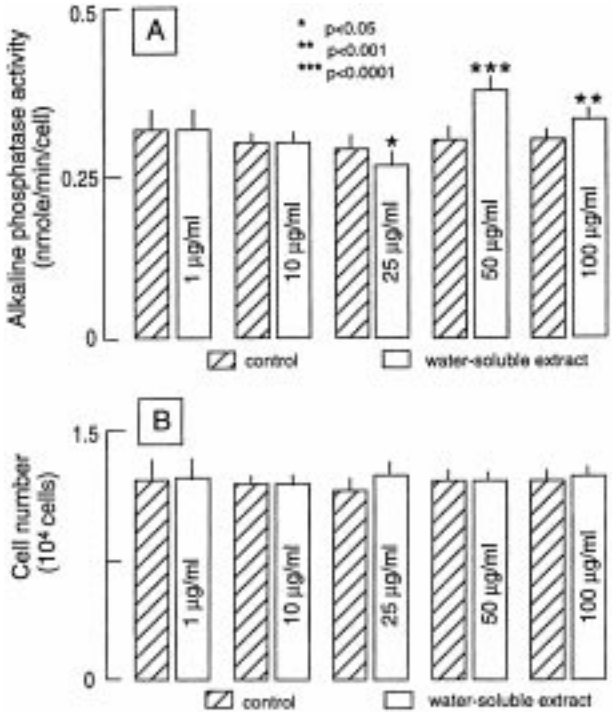


Figure 1 Effects of water-soluble extracts of nacre on alkaline phosphatase activity (A) and cell proliferation (B). Confluent osteoblasts were treated for 48 h with water-soluble extract or its vehicle (DMEM). Results are the mean ± SE, $n = 120$.

3.2. Effects of nacre extract on Bcl-2 concentration in proliferating and differentiating osteoblasts

Bcl-2 immunoreactivity was detected in the cytoplasm of control, untreated cells at every stage of the culture (Figs 3, 5 and 8). The intensity of immunostaining in the cytoplasm decreased from proliferating to subconfluent cells and was very low in confluent cells. The cytoplasm of cells incubated with nacre extract was strongly immunostained in proliferating cells (Figs 4 and 9) and also in subconfluent (Fig. 6) and confluent (Fig. 7) cells. The nuclei also contained intense brown immunostaining at all stages of culture (Figs 4, 6, 7 and 9). Some cells had two highly immunoreactive nuclei at the end of mitosis, before cytodieresis (Fig. 9). The nucleoli in the immunoreactive nuclei were also deeply immunostained (Fig. 10). The chromosomes in some cells were immunostained (Fig. 10). Fig. 7 shows confluent, mature osteoblasts with cell-cell contacts that have immunostained cytoplasm and stained nuclei in the center, and isolated cells at the periphery of the culture are still proliferating and have intensively immunoreactive cytoplasm and nuclei (Fig. 7). Cells incubated with non-immune serum in place of the primary antibody showed no immunostaining (not shown).

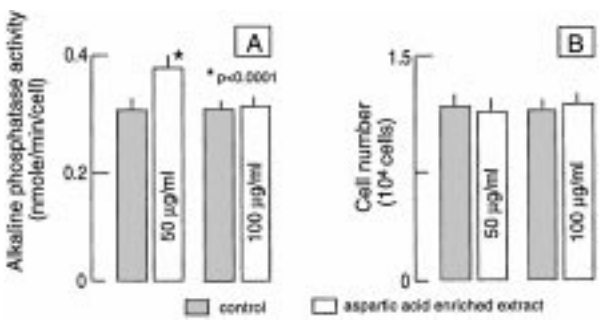


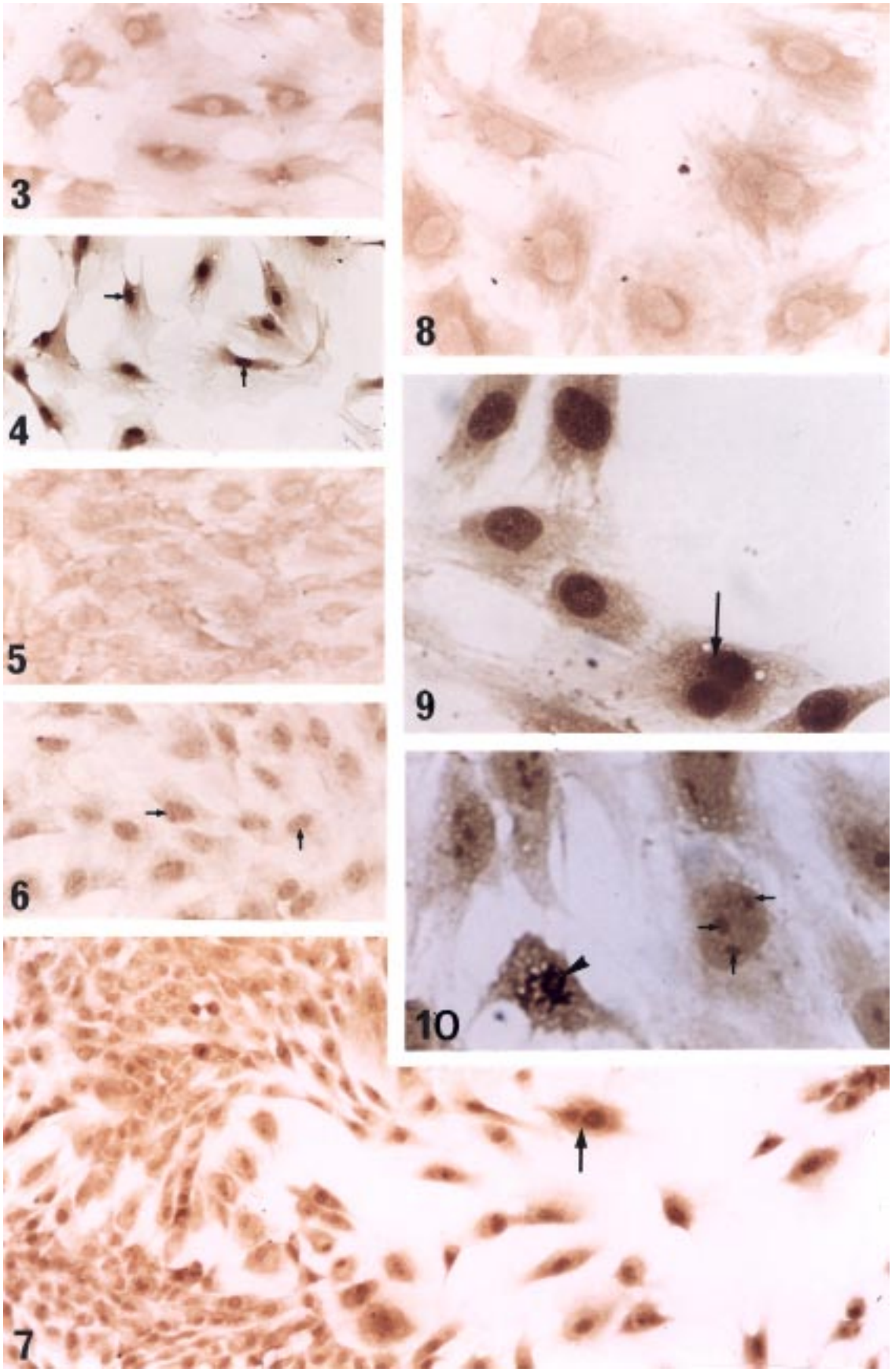
Figure 2 Effects of various concentrations of a fraction with high aspartic acid content (enriched fraction) of extracts of nacre on alkaline phosphatase activity (A) and cell proliferation (B). Confluent osteoblasts were treated for 48 h with aspartic acid-enriched extract of nacre or vehicle (DMEM). Results are means ± SE, $n = 60$.

4. Discussion

The alkaline phosphatase activity and Bcl-2 content of cultured osteoblasts from neonatal rat calvariae are significantly affected by nacre extract from Pinctada maxima. The water-soluble nacre extract appears to mediate the development of the mature osteoblast phenotype and stimulate the differentiation of osteoblastic cells in primary culture.

4.1. Stimulation of alkaline phosphatase activity

Nacre extract added to the medium of confluent osteoblasts having their maximal mature phenotype and the highest basal ALP activity caused a dose-dependent response. An extract concentration of 25 µg protein/ml caused a significant decrease in ALP activity (8%, $p < 05$), but 50 µg protein/ml caused a significant increase in ALP activity (20 fold, $p < 0.0001$) and 100 µg protein/ml increased ALP activity 7 fold ($p < 0.001$). These results cannot be due to differences in the proliferative state of the cells since they were obtained with confluent cells. Cells treated with high aspartic acid content fraction of nacre extract also showed a significant increase (23%, $p < 0.001$) ALP activity when incubated with 50 µg protein/ml. This result is very similar to the ALP response to 100 µg protein/ml water-soluble extract. These effects could be due to the extract of nacre providing factors to supplement those provided by the culture medium, with their action being protein dose-dependent. High ALP activity is generally considered to be one of the main markers of the osteoblast phenotype. The responsiveness of osteoblasts population to hormones has been shown to vary. The calciotropic hormone, 1,25 $(OH)_2$ vitamin D_3 stimulates or inhibits ALP activity in human bone-derived cells *in vitro* [22], in osteosarcoma cells [23], Saos cells [24] and in rat osteoblastic cells [25]. The *in vitro* results depend on how the hormone is delivered, short treatment or a continuous treatment [26]. A short treatment (48 h) significantly increased (50%) the alkaline phosphatase activity in confluent cultures of human osteoblastic cells [16]. The genomic effect of 1,25 $(OH)_2D_3$ are also dependent on the developmental stage of the osteoblast lineage [27]. These authors showed that the hormone stimulate mature osteoblasts, but inhibits immature osteoblasts. Our results show that the ALP activity of confluent cultures is affected by changes in nacre extract concentration, and finally a stimulation by extracts with concentrations of 50–100 µg protein/ml. Ruttner *et al*. [16] found that a change in the culture conditions of confluent cultures of human osteoblasts



Figures 3–10 Bcl-2 immunolocalization in primary cultures of osteoblasts from neonatal rat calvariae: untreated (3, 5, 8) and nacre water-soluble treated extract (4, 6, 7, 9, 10) cultures. 3, 4, 8, 9 = 3 days in culture, proliferative; 5, 6 = 6 days, pre-confluent; 7, 10 = 9 days, confluent.

(MEM-10% calf serum instead of MEM-Ham F 12) reduced the production of ALP activity of osteoblasts and the cells became fibroblast-like. The active factors in the total water-soluble extract are still unknown, but we obtained a similar response and ALP activity stimulation similar to those detected by adding the fraction with a

high aspartic and glutamic acid side chains participate directly in calcium-binding in the shell of the mollusc, Haliotis rufescens [28], and nacrein is the major aspartic acid-rich calcium-binding protein in the nacreous layer of the oyster pearl [11]. Belcher *et al*. [29] reported that the macromolecules extracted from the nacreous shell layers cause aragonite formation *in vitro* and that carbonic anhydrase catalyses the formation of HCO_3^- thus participating in calcium carbonate crystal formation in the nacreous layer. Among many factors involved in osteoblasts differentiation, bone morphogenetic proteins (BMPs) have the most potent stimulatory activity [30]. The osteogenic growth factor OP–1 enhanced cellular ALP activity in primary cultures of RC cells in a concentration dependent manner [31]. The present data show that both the total water-soluble extract and the fraction with high aspartic acid content strongly stimulate the alkaline phosphatase activity in primary cultures of confluent osteoblasts.

4.2. Bcl-2 in the cytoplasm and nuclei

Control, untreated cultures of osteoblasts containing Bcl-2 only in the cytoplasm and the nuclei are unstained, at all stages of culture. The intensity of cytoplasmic immunostaining decreased to become very low when there was cell-cell contacts at confluence. Cultures containing nacre extract had greater ALP activity and the amount of Bcl-2 in the cytoplasm of all osteoblasts, at every stage of culture, was greatly increased. This intense cytoplasmic immunostaining for Bcl-2 suggests that the extract acts on osteoblasts by supplementing the culture medium with factors contained in the water-soluble extract. The stimulation of Bcl-2 synthesis by nacre extract occured at the same concentration of extract proteins as the stimultation of ALP activity (100 μg/ml). Thus, the water-soluble extract of nacre may have antiapoptotic action, via Bcl-2. The increased Bcl-2 in the cytoplasm was independent of the developmental stage. Bcl-2 inhibits programd cell death [32, reviewed in 33], maintains survival in several cell types *in vivo* [34] and blocks the apoptosis induced by several chemotherapeutic drugs and by Y-irradiation [35]. Bcl-2 has been found *in vivo* in osteoblasts and osteocytes [19] and it has been suggested that it is involved in the survival of chondrocytes and osteoblasts throughout their development. The temporal and spatial distribution of Bcl-2 in the osteoblasts and osteocytes of trabecular bone of the metaphysis in growing rats [36] shows that Bcl-2 is highly concentrated in the cytoplasm of osteoblasts and osteocytes, and suggests that Bcl-2 acts to ensure osteocytes survival. Nutrient limitation is one of the primary causes of cell death in culture. Bcl-2 substantially extends the duration of batch cultures by reducing the rate of cell death during the decline phase [37] and its overproduction enhanced survival of mammalian cells. Overproduction of Bcl-2 also prolongs the viable culture of hybridoma cells [38]. Thus, the present results indicating that the water-soluble extract of nacre caused overproduction of Bcl-2 in the cytoplasm of mature osteoblasts suggest that water-soluble nacre extract prolongs the life of mature osteoblasts and also enhances osteoblastic synthetic activity, as indicated by the high alkaline phosphatase activity.

The subcellular pattern of Bcl-2 distribution in cultured osteoblasts treated with the nacre extract showed that the nuclei were also intensely immunostained. Strong Bcl-2 immunoreactivity was found over the whole chromatin, in all the cells and at every stage of culture, from proliferative until confluence, in quiescent osteoblasts and at the end of mitosis, when one cell contains two nuclei, just before cytodieresis. Electron microscopic data indicated faint Bcl-2 immunolabeling in the nuclei of chondrocytes [19], patches of Bcl-2 protein reminiscent of nuclear pore complexes [39] and association of Bcl-2 with the nuclear envelope [40]. Thus, the presence of Bcl-2 in the nuclei of treated osteoblasts suggests that the extract leads to transport of the overproduced protein from the cytoplasm to the nuclei. There is now evidence that Bcl-2 is involved in cell proliferation [41]. We have found Bcl-2 over the chromosomes of mitotic osteoblasts in treated cultures. Bcl-2 was abundant during mitosis, in prophase over the chromosomes, and over the whole chromatin, after the reconstitution of the two nuclei, before cytodieresis and also after the separation of the daughter cells. Bcl-2 protein had also been found in the chromosomes of mitotic nuclei in cultured epithelial cell lines and correlated with the cell cycle [41]. Our results also show Bcl-2 immunostaining in the nucleoli of osteoblasts of proliferating cells in treated cultures, suggesting that Bcl-2 influences the transcriptional activity of the cell, so that the nacre extract may regulate gene transcription via Bcl-2. Lu *et al*. [41] showed that the synthesis and distribution of Bcl-2 by cultured epithelial cells is influenced by treatment with excess thymidine and that the high concentration of Bcl-2 in mitotic nuclei and chromosomes suggests a role for the protein in cell immortalization. The present data support the results of Lu *et al*. and suggest that the water-soluble nacre extract can help protect cells from apoptosis.

Our findings thus indicate that water-soluble nacre extracts influence the differentiation of osteoblasts and the survival of mature osteoblasts in culture. The extract causes confluent mature phenotype cells to maintain their osteoblastic features as judged by high alkaline phosphatase activity and the overproduction of cytoplasmic Bcl-2, an anti-apoptotic protein. The nacre extract also acts on mitotic processes, via nuclear Bcl-2, at all stages of culture. The extract may also act on the cell cycle, via the binding of Bcl-2 to chromosomes and influence gene transcription. Further studies are now needed to investigate the pleiotropic effects of water-soluble nacre extract on osteoblasts, and to elucidate the cellular and molecular mechanisms by which it acts on bone cells *in vitro*. This should provide a better understanding of how endoosseous implants of nacre, as blocks or as powder, stimulate the differentiation of bone cells and induce bone formation.

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References

1. E. LOPEZ, B. VIDAL, S. BERLAND, S. CAMPRASSE, G. CAMPRASSE and C. SILVE, *Tissue and Cell* **24** (1992) 667.
2. G. ATLAN, N. BALMAIN, S. BERLAND, B. VIDAL and E. LOPEZ, *C. R. Acad. Sci. Paris* **320** (1997) 253.
3. E. LOPEZ, S. BERLAND, A. LE FAOU, *Bull. Instit. Oceano. Monaco* **3** (1995) 49.
4. O.DELATTRE, Y. CATONNE, S. BERLAND, S. BORZEIX and E. LOPEZ, *Eur. J. Orthop. Surg. Traumatol.* **7** (1997) 15.
5. G. ATLAN, O. DELATTRE, S. BERLAND, A. LE FAOU, G. GABIAS, D. COT and E. LOPEZ, *Biomaterials* **20** (1999) 1017.
6. H. LIAO, C. BRANDSTEN, T. LUNDMARK and J. LI, *J. Mater. Sci. Mater: Med.* **8** (1997) 823.
7. P. WESTBROEK and F. MARIN, *Nature* **392** (1998) 861.
8. C. SILVE, E. LOPEZ, B. VIDAL, D. C. SMITH, S. CAMPRASSE, G. CAMPRASSE, and G. COULY, *Calcif. Tissue Int.* **51** (1992) 363.
9. E. LOPEZ, M. GIRAUD, S. BERLAND, A. LE FAOU, C. MILET and G. GUTIERREZ, Brevet n° FR 95 15650.
10. J. P. CUIF and Y. DAUPHIN, *Aquat. Living Resource* **9** (1996) 187.
11. H. MIYAMOTO, T. MIYASHITA, M. OKUSHIMA, S. NAKANO and T. MORITA, *Proc. Natl. Acad. Sci. USA* **93** (1996) 9637.
12. S. WEINER and L. HOOD, *Science* **190** (1975) 987.
13. C. G. BELLOW, J. E. AUBIN, J. N. M. HEERSHE and M. E. ANTOSZ, *Calcif. Tissue Int.* **38** (1986) 143.
14. G. S. STEIN, J. B. LIAN and T. A. OWEN, *Faseb J.* **4** (1990) 3111.
15. J. E. AUBIN, K. TURKSEN and J. N. M. HEERSCHE in ''Cellular and Molecular Biology of Bone'', edited by M. Noda (Academic Press, San Diego, 1993) p. 1.
16. A. RUTTNER, O. SABIDO, C. MASSOUBRE, F. RASCLE and J. FREY, *In vitro Cell Dev. Biol.-Animal* **33** (1997) 757.
17. G. WONG and D. V. COHN, *Nature* **252** (1974) 1811.
18. M. LIEBEHERR, B. GROSSE, M. KACHKACHE and S. BALSAN, *J. Bone Miner. Res.* **8** (1993) 1365.
19. Y. WANG, R. TOURY, M. HAUCHECORNE and N. BALMAIN, *Histochem. Cell Biol.* **108** (1997) 45.
20. F. MOUTAHIR-BELQASMI, Y. WANG, M. LIEBERRHER, R. TOURY, E. LOPEZ and N. BALMAIN, Second French Days of Biology of Mineralized Tissues, *INRA, Versailles, 1999*, in preparation.
21. R. GOLDMAN and Z. BAR-SHAVIT, *J. Natl. Cancer Inst.* **63** (1979) 1009.
22. J. N. BERESFORD, I. A. GALLAGHER and R. G. G. RUSSELL, *Endocrinology* **119** (1986) 1176
23. A. E. GRIGORIADIS, P. M. PETKOVICH, E. E. ROSENTHAL and J. N. M. HEERSCHE, *Endocrinology* **119** (1986) 932.
24. L. G. RAO, M. K. SUTHERLANS, G. S. REDDY, M. L. SIU-CALDERA, M. R. USKOKOVIC and T. M. MURRAY, *Bone* **19** (1996) 621.
25. V. H. SPIESS, P. A. PRICE, J. L. DEFTOS and S. C. MANOLAGAS, *Endocrinology* **118** (1986) 1340.
26. T. A. OWEN, M. ARANOW, V. SHALHOUB, L. M. BARONE, L. WILMING, M. S. TASSINARI, M. B. KENNEDY, S. POCKWINSE, J. B. LIAN and G. S. STEIN, *J. Cell Physiol.* **143** (1990) 420.
27. L. C. GERSTENFELD, D. ZURAKOWSKI, J. L. SCHAFFER, D. P. NICHOLS, C. D. TOMA, M. BROESS, S. P. BRUDER and H. J. CAPLAN, *Endocrinology* **137** (1996) 3957.
28. M. A. CARIOLOU and D. E. MORSE, *J. Physiol. B* **157** (197) 717.
29. A. M. BELCHER, X. H. WU, R. J. CHRISTENSEN, P. K. HANSMA, G. D. STUCKY and D. E. MORSE, *Nature* **381** (1996) 56.
30. Y. WADA, H. KATAOKA, S. YOKOSE, T. ISHIZUYA, K. MIYAZONO, Y. M. GAO, Y. SHIBASAKI and A. YAMAGUCHI, *Bone* **22** (1998) 479.
31. A. M. KITTEN, J. C. LEE and M. S. OLSON, *Am. J. Physiol.* **32** (1995) E 918.
32. S. J. KORSMEYER, *Blood* **80** (1992) 879.
33. Y. L. HUANG and C. K. CHOU, *J. Biomed. Sci.* **5** (1998) 185.
34. J. R. PARK and D. M. HOCKENBERY, *J. Cell Biochem.* **60** (1996) 12.
35. J. C. REED, *J. Cell Biol.* **124** (1994) 1.
36. N. BALMAIN, R. TOURY, Y. WANG, F. MOUTAHIR-BELQASMI and E. LOPEZ, *Bone* **20** (1997) A 142.
37. R. P. SINGH, A. N. EMERY and M. AL-RUBEAI, *Biotechnol. Bioeng.* **52** (1996) 166.
38. Y. ITOH, H. UEDA and E. SUZUKI, *Biotechnol. Bioeng.* **48** (1995) 118.
39. S. KRAJEWSKI, S. TANAKA, S. TAKAYAMA, M. J. SCHIBLER, W. FENTON and J. C. REED, *Cancer Res.* **53** (1993) 4701.
40. D. M. HOCKENBERG, Z. N. OLTVAI, X. M. YIN, C. L. MILLIMAN and S. J. KORSMEYER, *Cell* **75** (1993) 241.
41. Q. L. LU, A. M. HANDY, M. A. HAJIBAGHERI, S. E. GSCHMEISSNER, J. TAYLOR-PAPADIMITRIOU, S. KRAJEWSKI, J. C. REED and N. A. WRIGHT, *J. Cell Science* **107** (1994) 363.

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