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STIMULATORY EFFECTS OF CREATINE ON METABOLIC ACTIVITY, DIFFERENTIATION AND MINERALIZATION OF PRIMARY OSTEOBLAST-LIKE CELLS IN MONOLAYER AND MICROMASS CELL CULTURES

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Abbreviations used

ALP: alkaline phosphatase; **BMD**: bone mineral density; **CK**: creatine kinase; **Cr:** creatine; **GPA**: b-guanidinopropionic acid; **MTT**: 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyltetrazoliumbromide; **NR**: neutral red; **PCr**: N-phospho-creatine; **TEM**: transmission electron microscopy

Abstract

The effects of creatine (Cr) supplementation on primary rat osteoblast-like cells cultured as monolayer and micromass were investigated. Cr was added to the medium at concentrations of either 10 or 20 mM. At various time points, the cell cultures were analyzed morphologically, metabolically and biochemically.

The degree of differentiation of primary osteoblast-like cell cultures was higher in micromass cultures compared to monolayer cultures, as judged by alkaline phosphatase (ALP) activity and extent of mineralization. In both culture systems, Cr supplementation showed positive effects, which were dependent on the organizational level of the osteoblast-like cells in such a way that the cells in monolayer culture showed significantly increased metabolic activity, ALP activity and mineralization in the presence of Cr than without the supplement. In micromass cultures, Cr also significantly enhanced ALP activity and mineralization, without affecting metabolic activity. The effect of Cr on ALP activity was more pronounced at higher concentrations of Cr, but 20 mM Cr already showed some adverse effects on cell viability. In conclusion, chemically pure Cr added to low serum cell culture medium has a stimulatory effect on metabolic activity, differentiation and mineralization of osteoblast-like cells indicating that Cr supplementation could also be used as a potential clinical intervention to stimulate cell growth, differentiation and mineralization during bone repair *in vivo*.

Key Words: Osteoblast-like cells, creatine, viability, metabolic activity, ultrastructure, differentiation, mineralization, monolayer culture, micromass culture, cell protection.

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Introduction

Creatine (Cr) occurs naturally in the human body and is partly synthesised by the kidney, pancreas and liver (approximately 1-2 grams per day), and partly ingested with food (approximately 1-5 grams per day), especially with meat and fish (for review see Wyss and Wallimann, 1994; Wyss and Kaddurah-Daouk, 2000). Cr and the enzyme Cr kinase (CK) occur mainly in skeletal and heart muscle, brain, retina and testes (Wallimann and Hemmer, 1994), but are also present in smooth muscle, growing bone and cartilage, as well as in immune cells (Wallimann, *et al.*, 1992). Cr is taken up into these cells by a Cr transporter (CreaT) (for review see Guerrero-Ontiveros and Wallimann, 1998; Wyss and Kaddurah-Daouk, 2000). Within the cell, a phosphate group from ATP is transferred to Cr with the help of CK, thus forming high-energy phospho-Cr (PCr). The large pool of PCr acts as an energy storage to resynthesise ATP (adenosin-triphosphate) from ADP (adenosin-diphosphate). Besides its function as a temporal energy buffer, PCr also acts as spatial energy buffer to shuttle high-energy phosphates between mitochondria and cellular ATP utilisation sites (Wallimann *et al.*, 1992; Wyss and Wallimann, 1994).

For the development of normal bone and cartilage and during bone repair, cells require energy to survive, proliferate, differentiate and synthesize extracellular matrix, which then mineralizes. During these processes, ATP is generated by glycolysis, oxidative phosphorylation, as well as via the CK/PCr system present in these cells (Sjovall and Hansson, 1971; Somjen *et al.*, 1984c); for review see Wallimann and Hemmer (1994). Thus, CK is not only present in excitable tissues, like muscle, heart and brain cells, where the CK/PCr system plays an important role for cellular bioenergetics (Wallimann *et al.*, 1992), but is also present in cartilage and bone (Granstrom, 1986; Funanag *et al.*, 1992; Rajpurohit *et al.*, 1998). Dependent on the developmental stage of cartilage and bone, different CK isoforms are expressed at various levels, indicating an important role of CK and PCr for cartilage and bone development, differentiation and function in general (Katoh *et al.*, 1991; Pollesello *et al.*, 1991; Kvam *et al.*, 1992; Shapiro *et al.* 1992; Wallimann and Hemmer, 1994; Hobson *et al.*, 1999).

Resting and hypertrophied cartilage both contain PCr in the range of 0.1 to 0.4 nmole/µg DNA (Shapiro *et al.*, 1992), but the highest amount of PCr (around 1.8 nmole/µg DNA) is found in the proliferative zone of cartilage. In the transition zone of calcified cartilage and bone, no PCr is detectable. On the other hand, the highest content of Cr is found in resting cartilage (around 34 µmole/µg DNA), whereas the other zones all contain lower amounts of Cr (around 14-18 µmole/µg DNA) (Katoh *et al.*, 1991; Shapiro *et al.*, 1992). Chondrocytes undergoing hypertrophy *in vitro* and *in vivo* show an increase in CK activity (Katoh *et al.*, 1991; Shapiro *et al.*, 1992; Hobson *et al.*, 1999). There is also an increase in CK activity from resting-proliferative cartilage to hypertrophic cartilage (6 fold) and the transition zone of calcified cartilage and bone (17 fold) (Katoh *et al.*, 1991; Shapiro *et al.*, 1992). In addition, during cartilage calcification, there is an elevation in PCr turnover because the PCr concentration in calcified cartilage is low and the activity of the CK in this zone is high (Shapiro *et al.*, 1992). Thus, CK activity seems to be required for matrix synthesis and mineralization in the growth plate cartilage (Funanage *et al.*, 1992). There is evidence that chondrocytes and osteoblasts require large amounts of energy during mineralization (Cartier, 1969; Heyden and From, 1970; Kakuta *et al.*, 1986; Shapiro *et al.*, 1988; Pollesello *et al.*, 1991; Komarova *et al.*, 2000). In addition, maximal activity of ATPases is observed at the onset of matrix deposition, followed by a decrease of enzyme activities during the transformation of osteoblasts to mature osteocytes and at the time of chondrocytes hypertrophy (Heyden and From, 1970). During bone development, CK enzyme activity peaks in diaphyseal bone and cartilage in rats of peripubertal age (Somjen *et al.*, 1994). In bone, similar to cartilage, CK is also experimentally increased both *in vitro* and *in vivo* by insulin growth factor I (Somjen and Kaye, 1994); by 1,25dihydroxyvitamin D_3 (Somjen *et al.*, 1984a; Somjen *et al.*, 1984b); by parathyroid hormone (PTH) (Somjen *et al.*, 1985a; Somjen *et al.*, 1985b; Somjen *et al.*, 1987; Kaye *et al.*, 1990); by protease-resistant variants of PTH (Somjen *et al.*, 1995); by prostaglandin E_2 (Somjen *et al.*, 1985a; Somjen *et al.*, 1985b); by 17b-estradiol (Gray, 1989; Kaye *et al.*, 1990). Furthermore, the stimulation of bone cell energy metabolism by 17b-estradiol and testosterone is sex specific (Somjen *et al.*, 1991). Interestingly, the amount of bone produced during heterotropic bone formation by implantation of bone morphogenetic protein into the muscle of rats shows an almost parallel relationship to the level of CK (Ono *et al.*, 1994).

Cr supplementation, first employed by athletes to enhance muscle mass, power and performance (Mujika and Padilla, 1997), is increasingly recognized as a potentially important adjuvant therapeutic agent for patients with different muscle diseases, like muscle dystrophy, muscle disuse atrophy, as well as other neuromuscular and neurodegenerative diseases, like ALS, Huntington etc. (Tarnopolsky and Parise, 1999; Vorgerd *et al.*, 2000; Walter *et al.*, 2002; Bender *et al.*, 2005) (for reviews see Wallimann *et al.*, 1999; Wyss and Kaddurah-Daouk, 2000; Wyss and Schulze, 2002; Baker and Tarnopolsky, 2003; Persky *et al.*, 2003). Cr supplementation increases not only the pool of free Cr, but also that of PCr (Ipsiroglu *et al.*, 2001). Positive effects of Cr on regaining muscle mass and strength have been reported in rehabilitation of human subjects after immobilization (Hespel *et al.*, 2001). Furthermore, in a recent double-blinded clinical study with young patients suffering from muscular dystrophy, the bone mineral density increases on average by 3% and bone resorption was reduced by 30% as a "side effect" in the Cr treated group (Louis *et al.*, 2003). This shows that Cr may have direct beneficial effects on BMD (bone mineral density) or indirectly by affecting bones by increasing muscle strength.

In an attempt to show whether the energy stored in PCr could be used directly for bone mineralization, very crude preparations of PCr or mixtures of PCr and Cr at concentrations of 10^{-4} M have been added earlier directly to cultured embryonic chicken femora, a treatment which suggested a stimulatory effect on the mineralization of cartilage matrix in the epiphysis and the perichondrium of the diaphysis of these femora (Saito, 1959). In addition, PCr seemed to promote mineralization in differentiating chick limb bud mesenchymal cells in micromass cultures at a concentration of 2 mM (Boskey *et al.*, 1994).

Therefore, we wanted to study the effects of supplementing the culture media with chemically pure Cr on proliferation, differentiation and mineralization of osteoblast-like cells in monolayer and micromass cultures under controlled conditions.

The parameters investigated were viability based on the physical uptake of neutral red (NR) and metabolic activity by MTT (3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyltetrazoliumbromide). The MTT assay and the neutral red uptake are widely accepted to assess viability and growth of cells including osteoblasts in biocompatibility and cytotoxicity studies (Mosmann, 1983; Fini *et al.*, 2001; Ramires *et al.*, 2001; Aldini *et al.*, 2002; Ciapetti *et al.*, 2002; Yang *et al.*, 2002; Torricelli *et al.*, 2003). Differentiation was studied by morphological parameters such as histochemical staining for alkaline phosphatase (ALP) activity and degree of mineralization, as well as ultrastructure observed by transmission electron microscopy (TEM). As biochemical parameters ALP activity, DNA and protein content were measured at different time points in cell cultures with and without the addition of Cr.

Materials and Methods

Materials

All tissue culture disposable materials were purchased from Falcon (Becton Dickinson AG, Basel, Switzerland). Fetal calf serum and growth media were purchased from Gibco (Invitrogen, Basel, Switzerland). All chemicals were purchased from Fluka (Buchs, Switzerland), when not stated otherwise.

Methods

Enzymatic cell isolation. Parietal and frontal calvariae (4 per animal) were explanted aseptically from 6 day old IcoIbm rats. The calvariae were placed in Tyrode's

buffered salt solution calcium- and magnesium-free (TBSS). The periosteum and endosteum were removed enzymatically by treatment in 0.05 % trypsin (1:250; Sigma, Buchs, Switzerland) and 0.02 % collagenase A (0.76U/mg; Roche Diagnostics, Rotkreuz, Switzerland) dissolved in TBSS (40 calvaria/20 ml). The calvariae were shaken for 70 minutes in a water bath at 37°C. They were washed with TBSS and then transferred to 60 mm culture dishes (40 calvariae/dish) containing 5 ml of 0.02 % collagenase A (0.76 U/mg) in culture medium BGJ_b Fitton Jackson modification and placed in the incubator for 3 hours. After the second collagenase treatment the remaining calvariae were washed with culture medium supplemented with 10 % fetal calf serum (FCS). The calvariae were transferred into 60 mm culture dishes (4 frontal and 4 parietal/60 mm dish). The growth medium, supplemented with 10% FCS and 50µg/ml ascorbate, was changed completely every 48 h. The explant cultures were kept for 3 weeks. After 3 weeks the migrated cells, along with the calvariae, were treated with enzymes to harvest the cells. The dish was washed with TBSS and 5 ml of TBSS containing 0.05 % trypsin and 0.02 % collagenase A (0.76 U/mg) was added. After 1 hour in the incubator, the dish was washed with culture medium supplemented with 10 % FCS. The cells obtained were filtered through a 40 µm nylon mesh to remove bone debris and cell aggregates. The suspended cells were centrifuged at 600 g for 5 minutes. The cell pellet was resuspended in serum containing medium and centrifuged. The viability of the resuspended cells was examined by the 'dye exclusion' of 0.4 % trypan blue and the vital cells counted using a haemocytometer. The inoculation densities were $2 \cdot 10^5$ cells/ 10 cm^2 for monolayer and $2 \cdot 10^5$/30 µl/10 cm^2 for micromasses for all performed experiments. The micromass cultures were kept for 30 minutes in the incubator before 2 ml growth medium was added. After 4 days, the growth medium was completely removed. The control groups were kept in 4 ml BGJ_b supplemented with 1% FCS and 50 µg/ ml ascorbate. In the experimental groups, the growth medium was additionally supplemented with either 10 mM Cr or 20 mM Cr. All the cultures were kept at 37°C in a humidified atmosphere of 5 % CO_2 and 95 % air. All culture media were supplemented with 50 µg/ml ascorbate. To increase plating efficiency, during cell isolation and inoculation, no ascorbate was used (Burks and Peck, 1979). No antibiotics, or antimycotics, nor b-glycerophosphate were added. The media (4 ml/35 mm culture dish) were changed completely twice a week.

Light microscopy. After 1,2 and 3 weeks the cultures were stained histochemically for the alkaline phosphatase, using the Sigma Kit no. 85L. As a result, an insoluble, visible blue pigment is formed at sites of phosphatase activity. The deposition of calcium phosphate, at 2 and 3 weeks, was determined by von Kossa staining. A photograph was taken from the center of each culture dish under a Wild M400 macroscope. The mineralization (black deposits) in an area of 123 mm^2 was quantified by a Kontron IMCO 1000 image analyser.

Transmission electron microscopy (TEM). At 3 weeks, the cell cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 20 min, postfixation in 1% OsO_4 in 0.1 M cacodylate buffer pH 7.4 for 1h at 4°C, then 2 % aqueous uranyl acetate for 1h at room temperature; dehydration in an ethanol series and embedding in LR White (London Resin Co, Basingstoke, U.K.). Ultrathin sections through the nodular area of monolayer cultures were cut with a Drukker Diamond knife (Drukker International, Cuijk, The Netherlands) on a LKB III Microtome (LKB, Bromma, Sweden) and stained with 2% uranyl acetate and lead citrate (Reynolds, 1963). Osmium rich regions, i.e., the nodules in the monolayer, and the centers of the micromass cultures, were selected respectively. The sections were examined using a JEOL (Tokyo, Japan) JEM 100CX transmission electron microscope operated at 100kV.

Viability. At 1, 2, and 3 weeks, the viability of the cells was determined by using the neutral red method (Lindl and Bauer, 1994). The cells were incubated in 2 ml/10cm^2 neutral red solution (0.5 mg neutral red /ml growth medium) at 37°C for 3h. After rinsing the cells with PBS, the dye was extracted by addition of 4 ml/10cm^2 extraction buffer (50% ethanol in 1 % acetic acid) on a shaker. The absorbance of the supernatant was read at 540 nm versus extraction buffer in a Lambda 12 spectophotometer from Perkin Elmer (Rotkreuz, Switzerland).

Metabolic activity. At 1, 2, and 3 weeks, the metabolic activity was analyzed by using the Boehringer 'Cell Proliferation Kit I (MTT)' (Roche Diagnostics) and DMSO (dimethylsulfoxide) as solvent (Hoffman, 1994). The MTT Stock (5 mg/ml in sterile PBS) from Boehringer was diluted 1:10 with complete growth medium and the cells were incubated in 2 ml/10cm^2 MTT solution at 37°C for 3h. Then, MTT crystals were resolved in 4 ml/10cm^2 dimethylsulphoxide (DMSO) on a shaker. The absorbance of the supernatant was read at 550 nm versus DMSO in a Lambda 12 spectophotometer.

Biochemistry. Whole cultures were washed at 1, 2 and 3 weeks with phosphate buffered saline (PBS) and 500 µl 0.25 M sucrose/35 mm dish was added. The cells and the matrix were scrubbed off the dish and transferred into a cryotube and frozen at -80°C. Immediately before the assays, the cells were thawed and sonicated (3 x 20s) at 70 W and 20 kHz on ice. The samples were centrifuged at 600 g for 7 min. to remove cell debris and the volume of the supernatant was determined. The samples were analyzed in a Perkin Elmer Lambda 12 Spectrophotometer, except for the DNA determination, which was obtained using a Hoefer TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Alkaline phosphatase activity (ALP). We used Sigma Kit no. 245 for the quantitative, kinetic determination of alkaline phosphatase activity.

Total protein. The Bio-Rad protein assay kit II (BioRad, Glattbrugg, Switzerland) was used with bovine serum albumin as standards.

DNA. A simple and rapid assay for quantitative DNA determination in crude homogenates was used (Labarca and Paigen, 1980). Each sample was diluted with 3 ml of phosphate buffered saline, pH 7.4 containing 2M NaCl (DPBS) and sonicated as described above. Then aliquots were mixed with 0.1 µg/ml Hoechst 33258 (Aventis, Strasbourg, France) in DPBS and the fluorescence was read in a Hoefer TKO 100 Mini-Fluorometer.

Statistics. To compare the effect of the various growth media, contrast analyses of variance models were evaluated. Main effects and interaction effects were examined by F-Tests. 'Least Squares Means' (LS) were calculated to yield average means accounting for the other variables in the model. LS Means were compared by using Tukey's multiple range test. QQ-Plots of the residuals and Tukey-Anscombe plots (residuals x predicted) were analyzed to check for normal distribution assumption.

Results

Viability and metabolic activity

Quantitative analysis of the NR uptake as measure of cell viability in monolayer cultures showed a small but significant ($p<0.05$) interaction effect (Fig. 1A), meaning that the effects of time and Cr treatment were not additive. There was an increase in the NR uptake in the control group and the 10 mM Cr group over culture time, which was less evident in the 20 mM Cr group. This was reflected by statistical analysis, where the control and the 10 mM Cr group had significantly ($p<0.0002$, $p<0.0015$ respectively) lower values at 1 week than 3 weeks. There was no significant difference in the control and the Cr treated groups, except the 20 mM Cr group, which had significantly ($p<0.008$) lower values than the control group at 3 weeks. Similar results were obtained in the micromass cultures, which also had significantly ($p<0.03$) higher values for NR uptake at 2 weeks as compared to 1 week, but the values were similar at 2 and 3 weeks (Fig. 1B). The 20 mM Cr group was significantly ($p<0.0002$, $p<0.002$

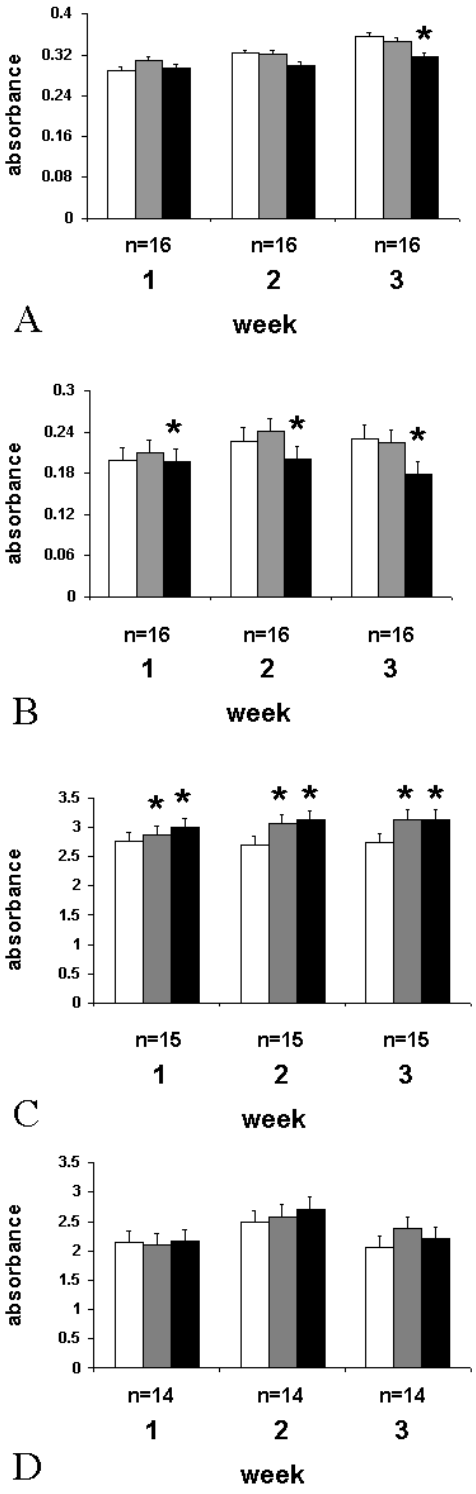

Figure 1. Quantitation of viability (NR) and metabolic activity (MTT) of osteoblast-like cells at 1, 2, and 3 weeks, cultured in the absence (control) and presence of 10mM or 20 mM of creatine (Cr). **A**: Viability by NR uptake in monolayer cultures. Note: significant ($p<0.05$) interaction effect, meaning that the effects of time and Cr treatment were not additive; control group and 10 mM Cr groups were significantly lower ($p<0.0002$ and $p<0.0015$, respectively) at 1 week than at 3weeks; control group significantly higher ($p<0.008$) than the 20 mM Cr group at 3 weeks. **B**: Viability by NR uptake in micromass cultures. Note: Values at 2 weeks significantly ($p<0.03$) higher than at 1 week; 20mM Cr values significantly lower ($p<0.0002$ and $p<0.002$) than control and 10 mM Cr group. **C**: Metabolic activity by MTT conversion in monolayer cultures. Note: control group was significantly lower ($p<0.0002$) than the both 10 and 20 mM Cr groups. **D**: Metabolic activity by MTT conversion in micromass cultures. Note: significantly ($p<0.0002$) lower values at 1 week and 3 weeks than at 2 weeks. Control cells grown in medium with low serum concentration (white columns). Cells grown under the same conditions plus 10 mM Cr (grey columns) and cells grown with supplementation of 20mM Cr (black columns). Data are expressed as least squares means + 1.96x standard error.

respectively) lower than both the control and the 10 mM Cr group, but the later ones were similar. These results with monolayer and micromass cultures demonstrate a significant increase in the number of viable cells during culture time and no negative effects on the viability by supplementation of the growth medium with 10 mM Cr, but the higher concentration of Cr at 20 mM had some adverse effects in both culture systems.

However, Cr revealed a clear positive treatment effect on the metabolic activity (MTT) in monolayer cultures (Fig. 1C). The control group was significantly ($p<0.0002$) lower than the both 10 and 20 mM Cr groups. There was no difference amongst the two Cr groups. The metabolic activity of the cells increased during culture time, but it was not significant. This was in contrast to micromass cultures, where the MTT activity peaked at 2 weeks, showing significantly ($p<0.0002$) higher values than at 1 week and 3 weeks (Fig. 1D). The values at 1 and 3 weeks were similar. The control was slightly lower than the Cr groups, but the difference was non significant. These results show that effect of Cr supplementation on the metabolic activity did dependent on the culture system used meaning that only in monolayer cultures a clear stimulatory effect of Cr on the metabolic activity could be demonstrated. No adverse effect of Cr on the metabolic activity could be detected in both culture systems. The addition of 20 mM Cr, however, clearly reduced the number of viable cells in both culture systems, but it still stimulated the metabolic activity in monolayer cultures, which was less evident in the micromass cultures.

Biochemistry

There was a non-significant increase in the DNA content in all groups over time in monolayer cultures (Fig. 2A). The control was lower than the Cr groups, but the differences were non significant. This was in contrast to micromass cultures, where the values for the DNA content were comparable in all groups at all time points (Fig. 2B). Comparison of monolayer and micromass cultures revealed significantly $p<0.0005$) higher values for monolayer than micromass cultures. So the addition of Cr had no adverse effects on the proliferation of the cells. Furthermore, proliferation of the cells was generally suppressed when the cells were cultured in an organoid system such as micormasses as compared to the standard monolayer cultures.

Differentiation of the cells in monolayer and micromass cultures, as measured by the ALP activity, significantly

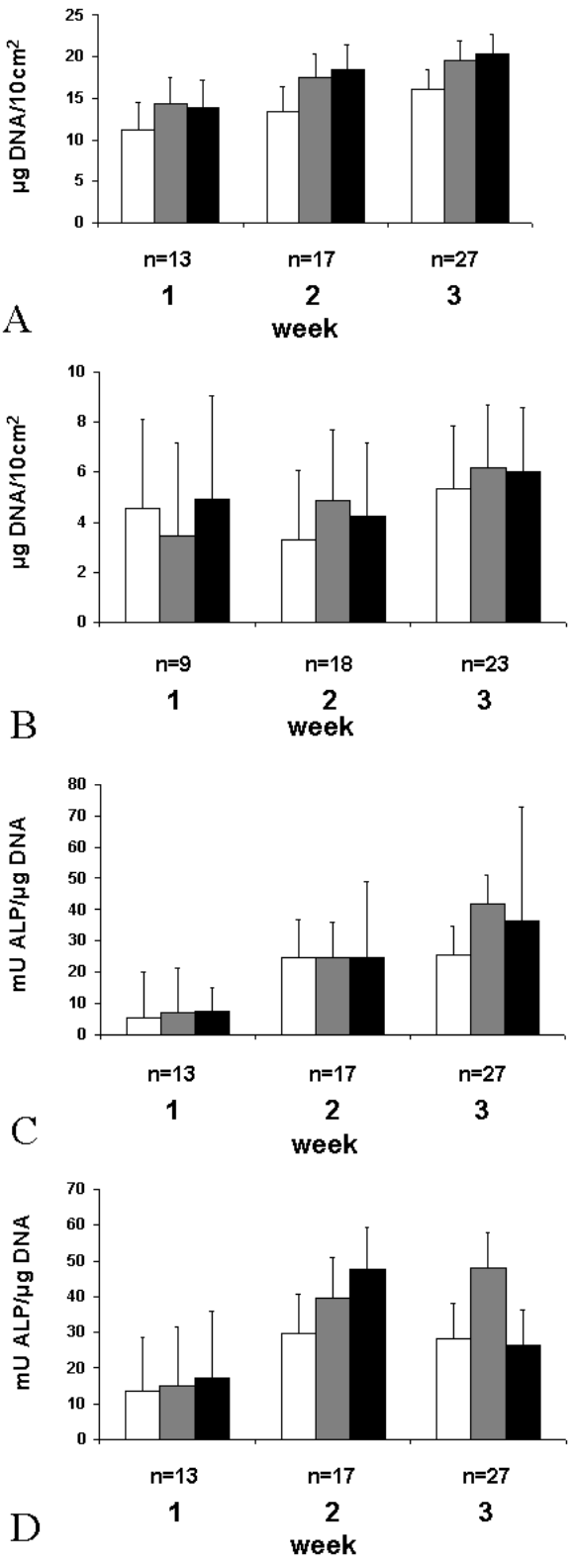

Figure 2. DNA content and ALP activity of osteoblast-like cells at 1, 2, and 3 weeks, cultured in the absence (control) and presence of 10mM or 20 mM of creatine (Cr). **A**: DNA content per dish in monolayer cultures. **B**: DNA content per dish in micromass cultures. Note: Monolayer significantly ($p<0.0005$) higher values in total DNA than micromass. **C**: ALP activity normalized to DNA content in monolayer cultures. **D**: ALP activity normalized to DNA content in micromass cultures. Note: significantly ($p<0.0005$) lower ALP activity in monolayer than in micromass; values for ALP activity at 2 and 3 weeks significantly higher ($p<0.0001$) than at 1 week; significant ($p<0.025$) stimulatory effect of 20 mM Cr as compared to control. Control cells grown in medium with low serum concentration (white columns). Cells grown under the same conditions plus 10 mM Cr (grey columns) and cells grown with supplementation of 20mM Cr (black columns). Data are expressed as least squares means + standard error**.**

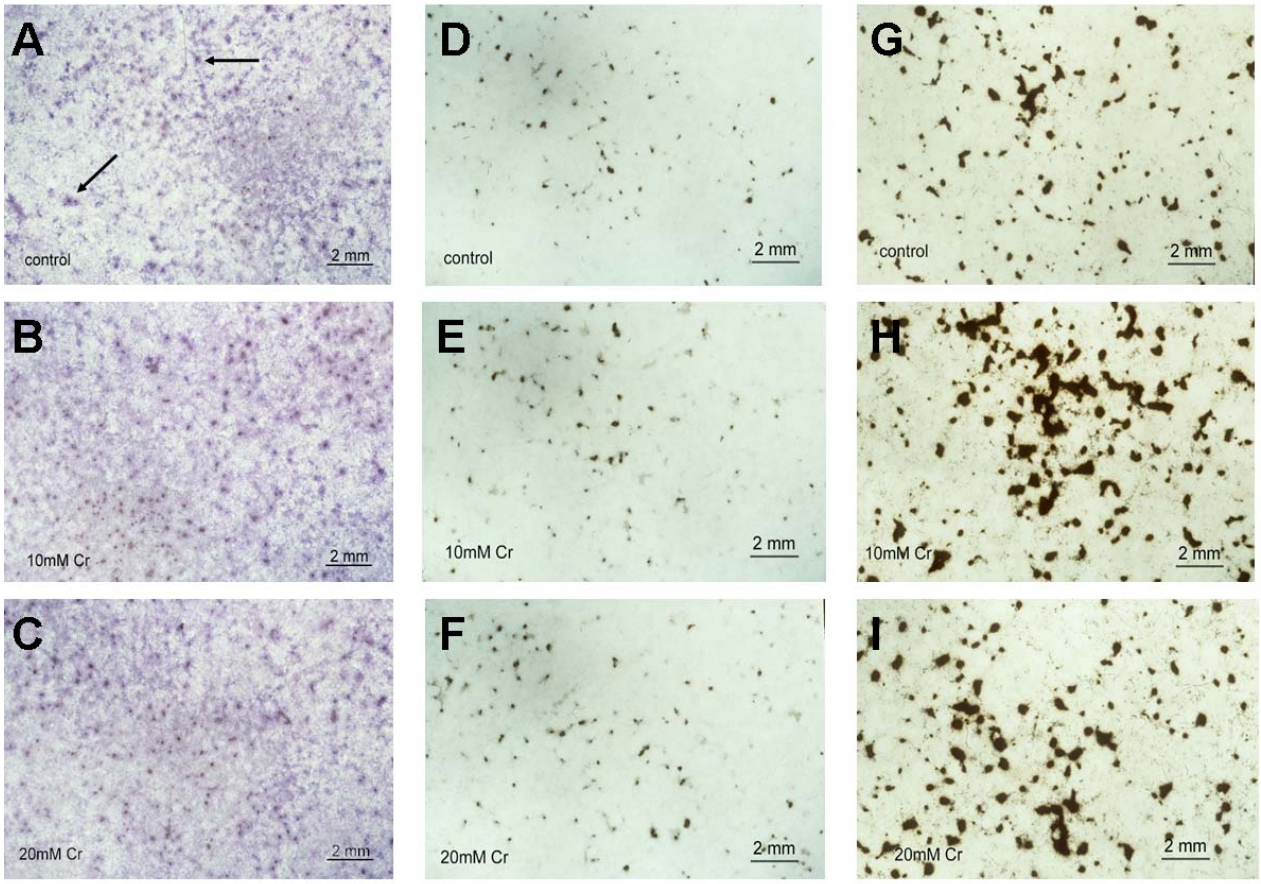

Figure 3. Histochemical staining of ALP activity and detection of calcification visualized by von Kossa staining in monolayer cultures of osteoblast-like cells at 2, and 3 weeks, cultured in the absence (control) and presence of 10mM or 20 mM of creatine (Cr). ALP activity is shown in blue and mineralization in black. There was no counterstaining of the cells. Note the staining is more pronounced in the Cr groups than in the control. Nodule formation (arrow). A: ALP activity of control group at 2 weeks. B: ALP activity of 10mM Cr group at 2 weeks. C: ALP activity of 20 mM Cr group at 2 weeks. D: Mineralization of control group at 2 weeks. E: Mineralization of 10mM Cr group at 2 weeks. F: Mineralization of 20 mM Cr group at 2 weeks. G: Mineralization of control group at 3 weeks. H: Mineralization of 10mM Cr group at 3 weeks. I: Mineralization of 20 mM Cr group at 3 weeks.

($p<0.0002$, $p<0.0001$) increased from the first to the second week and remained similar thereafter (Figs. 2C, D). At 1 and 2 weeks in monolayer, all groups were rather similar, but at 3 weeks, the Cr treated groups were higher than the control (Figure 2C). In micromass cultures at 2 weeks, there was a dose dependent increase in ALP activity from control to 10 and 20 mM Cr. At 3 weeks, only the 10 mM Cr group was higher than the control, whereas the control and the 20 mM Cr group had similar values. Statistical analysis showed that ALP activity was significantly ($p<0.0002$) lower at 1 week than at 2 and 3 weeks. In addition, there was an overall significant ($p<0.025$) stimulatory effect of 20 mM Cr as compared to control, whereas 10 mM Cr was comparable to the control. Furthermore, micromasses had significantly ($p<0.0005$) a higher ALP activity than monolayers. These results showed that the micromass system reduced the proliferation and increased the differentiation as compared to the standard monolayer system. In addition, the supplementation of the growth medium with 20 mM Cr had a stimulatory effect on the ALP activity, which is in accordance with the stimulatory effect on the metabolic activity. However, this high concentration had had an adverse effect on the viability.

Morphology

At 1 week nearly every cell under all conditions was histochemically positive for ALP activity (data not shown). The cells in monolayer cultures were confluent. There was no obvious difference visible between the control and the groups supplemented with Cr at 1 week (data not shown). At 2 weeks, the cells in monolayer (Fig. 3) and micromass cultures (Fig. 4) of all groups formed dense ALP positive and calcified nodules, which were more prominent at 3 weeks. In micromass cultures, the cells were migrating form the originally inoculated drop of cells towards the periphery of the culture dish (Fig. 4). In the control groups of both monolayer and micromass cultures the histochemical staining for ALP activity and calcification was less obvious than in the experimental groups at 2 and 3 weeks. Image analysis showed that both experimental groups of 10 and 20 mM Cr had significantly more calcified area in the monolayer and micromass cultures respectively, compared to the control group (Fig. 5). The

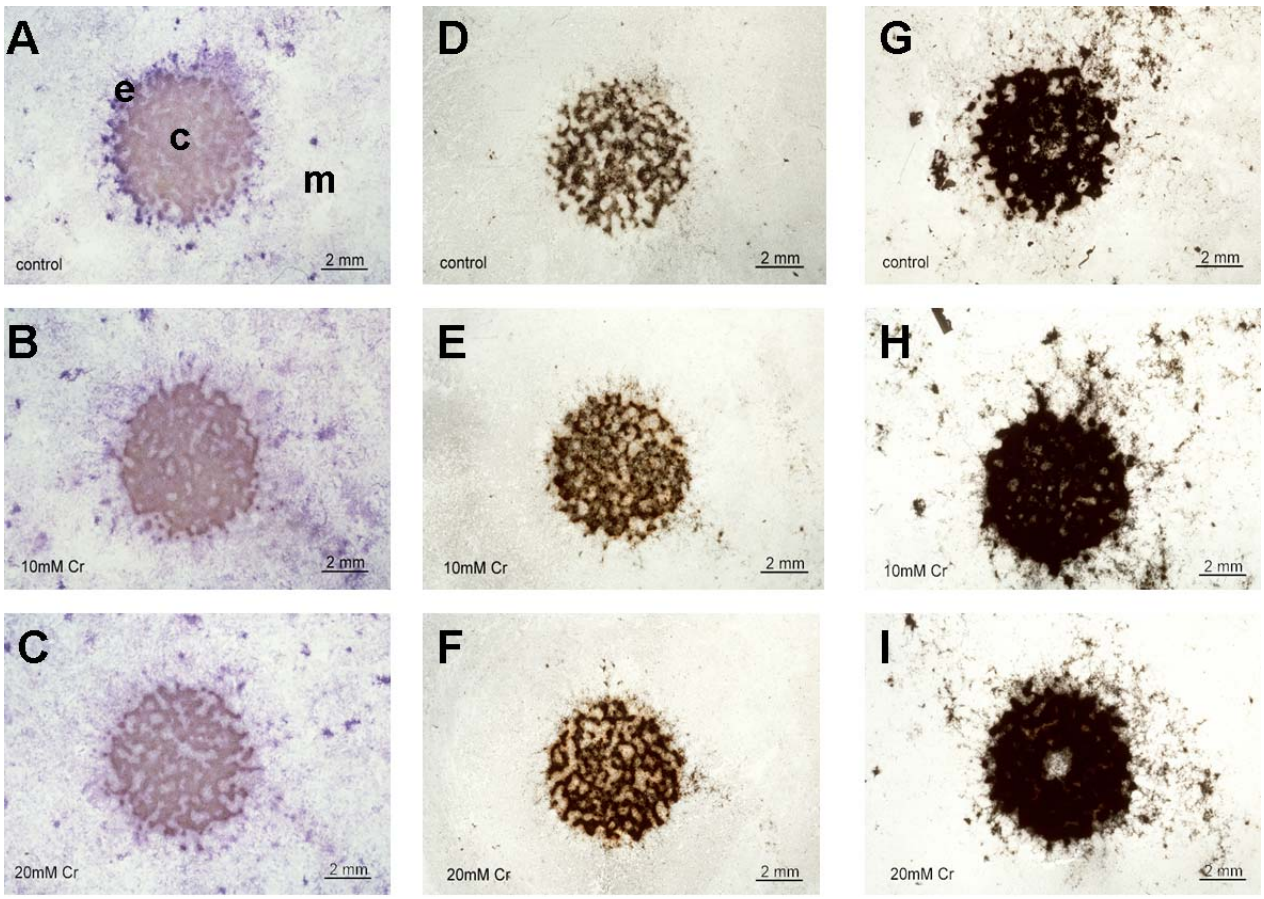

Figure 4. Histochemical staining of ALP activity and detection of calcification visualized by von Kossa staining in micromass cultures of osteoblast-like cells at 2, and 3 weeks, cultured in the absence (control) and presence of 10mM or 20 mM of creatine (Cr). ALP activity is shown in blue and mineralization in black. There was no counterstaining of the cells. Note the staining is stronger in the Cr groups than in the control. A: ALP activity of control group at 2 weeks. B: ALP activity of 10mM Cr group at 2 weeks. C: ALP activity of 20 mM Cr group at 2 weeks. D: Mineralization of control group at 2 weeks. E: Mineralization of 10mM Cr group at 2 weeks. F: Mineralization of 20 mM Cr group at 2 weeks. G: Mineralization of control group at 3 weeks. H: Mineralization of 10mM Cr group at 3 weeks. I: Mineralization of 20 mM Cr group at 3 weeks. The centre of the originally inoculated drop of cells (c) and border (e), cells migrating away from the centre (m) towards the periphery.

values of the control and the 10 mM Cr groups were slightly higher at 3 weeks than at 2 weeks, but the difference was non significant in monolayer cultures. In contrast, in micromass cultures all values significantly increased over time (Fig. 5B). Thus, the morphological and histochemical findings, showing a positive effect of Cr for ALP activity staining, were fully in agreement with the biochemical results (see above).

TEM-Ultrastructure

The ultrastructure of the cells in the nodular area was similar in all groups. In all groups there was collagen production and mineralization (Fig. 6). At the interface between the bottom of the culture dish and the first cell layer, some fuzzy electron dense material but hardly any collagen was visible (not shown). In this area, there were often some dead cells found in all groups (not shown).

The cells were elongated except for the cells in the top layer, which were oval in shape and often had cell blebs (not shown). The cells displayed many cell-cell contacts either as focal points or with longer cell membranes in very close apposition to each other, as well as many cell processes, which were mainly parallel to the culture dish surface (not shown). In addition, cells had a well developed rough endoplasmatic reticulum (rER), which was dilated in some cells and many free ribosomes (Fig. 7). Mitochondria were numerous and of various sizes and shapes, ranging from round to banana-shaped (Fig. 7). It was observed that the Cr groups on average showed structurally better preserved mitochondria (Fig. 7B, arrows) than the control group, where often swollen mitochondria could be seen (Fig. 7B, arrow heads). The Golgi area and the vesicles were also numerous. They were either small or large, of regular or irregular shape, and their contents were electron lucent or electron dense (Fig. 7). There were coated pino/exocytotic vesicles fused with the plasma membrane and the cytoskeleton consisted of many microfilaments (not shown). In many cells there was as a dense area of microfilaments running parallel to the plasma membrane (not shown).

The bulk of the collagen fibrils were found in the middle to upper cell layers (Fig. 6). They were often found in membrane folds within the cells or cell processes (Fig. 8). The diameters of the fibrils in the membrane folds and

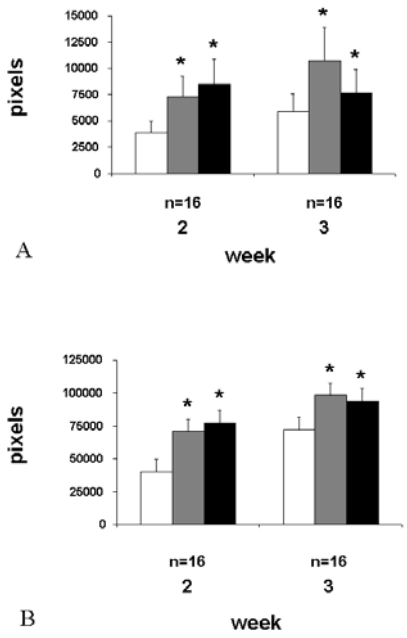

Figure 5. Quantification of mineralized area in monlayer and micromass cultures of osteoblast-like cells at 2 and 3 weeks, cultured in the absence (control) and presence of 10mM or 20 mM of creatine (Cr). A. Monolayer culture. Note: control significantly ($p<0.0015$, $p<0.0075$) less calcified area than 10 and 20 mM Cr groups. B. Micromass culture. Note: control significantly ($p<0.0001$) less calcified area than 10 and 20 mM Cr group; significant ($p<0.0001$) increase over time. Control cells grown in medium with low serum concentration (white columns). Cells grown under the same conditions plus 10 mM Cr (grey columns) and cells grown with supplementation of 20mM Cr (black columns). Data are expressed as least squares means + 1.96x standard error.

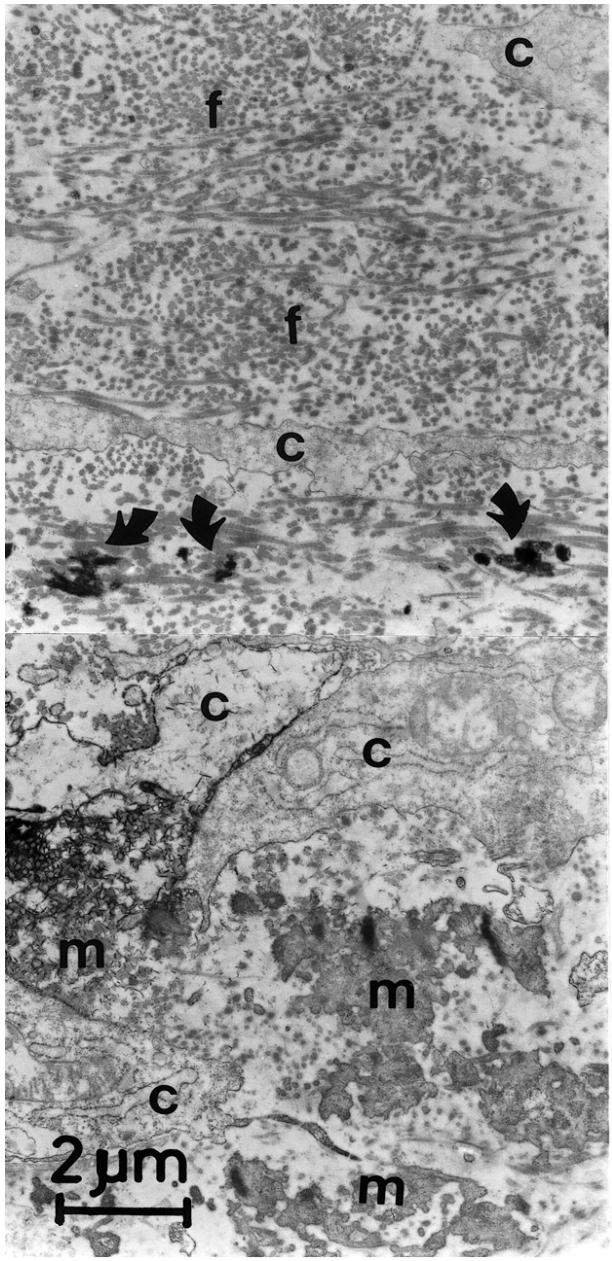

Figure 6. Ultrastructure of a mineralizing area in monolayer cultures. The cells (c) were surrounded by abundant collagen fibrils, which were orthogonally oriented (f). In the unmineralized area, there were single mineralized patches (arrow). Deeper in the matrix more heavily mineralized zones were evident (m).

the ones deeper in the matrix were rather similar (Fig. 8). The collagen fibrils were orthogonally oriented (Fig. 6; Fig. 8). Typically, they were either cut in cross sections or longitudinally. The characteristic cross-striation was clearly seen. The diameter of the fibrils was quite uniform in the unmineralized area (Fig. 6; Fig. 8). In the area of mineralization, the diameter of collagen fibrils varied and mineralized patches could be seen (Fig. 8). Matrix vesicles in the extracellular matrix were also found (Fig. 8).

No other obvious differences were observed between the control and experimental groups by conventional electron microscopy, although no quantitative statistical comparison was made at the ultrastructural level. However, the Cr groups tended to generally display better preserved mitochondria and more collagen fibrils within membrane folds than the control.

Discussion

This study showed that Cr had clear stimulatory effects on metabolic activity, differentiation of primary rat osteoblast-like cells, as well as on the mineral deposition by these cells. The extent and timing of this stimulation by Cr was, however, dependent on the cell culture system used, e.g. whether osteoblast-like cells were cultured as monolayer or as micromass, the concentration of Cr added to the growth medium, as well as the time in culture.

The cells were sensitive to the culture system used. In the micromass system cell proliferation was reduced but differentiation was promoted, as compared to the standard monolayer system under serum-reduced conditions. Similar observations were made in the presence of 10% fetal calf serum in the growth medium (Gerber and ap Gwynn, 2001; Gerber and ap Gwynn, 2002). Micromass culture represent an organoid culture system promoting the formation of cell-cell contacts at the onset of culture

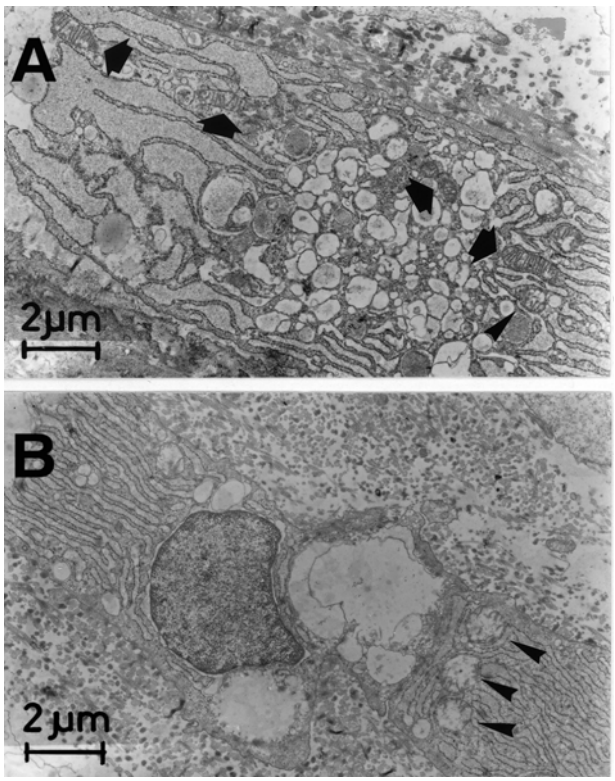

Figure 7 Ultrastructure of mitochondria in monolayer cultures. A: 10 mM Cr group with well preserved mitochondria (arrow) and other mitochondria (arrow head). B: Swollen mitochondria in the control group (arrow head).

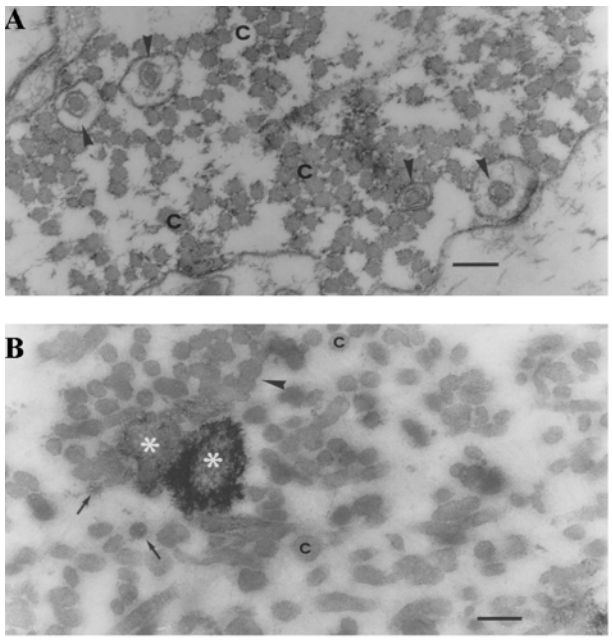

Figure 8. Ultrastructure of extracellular matrix in monolayer cultures**.** A: 10 mM Cr group. Collagen fibrils in membrane folds (arrow) have a similar diameter as the collagen fibrils in the matrix (c). B: control group. Mineralized patches (star) within collagen fibrils (c), which had a variable diameter and some were in very close contact (arrow head) and matrix vesicles (arrows). Bar=150 nm.

and this resembles in many aspect the condensation process observed during intramembranous bone formation *in vivo* where mesenchymal progenitors condense and directly differentiate into osteoblasts (Hall, 1987).

There were no differences observed in the ultrastructure of the cells when they are kept under low serum concentration as compared to the addition of 10 % FCS (Gerber and ap Gwynn, 2001; Gerber and ap Gwynn, 2002). Additionally, many collagen fibrils were found within membrane folds, which resembled the extracellular fibrils in diameter, staining pattern, and electron density. These are also previously described in rat osteoblasts (Gerber and ap Gwynn, 2002) as well as in tendon fibroblasts (Trelstad and Hayashi, 1979).

Supplementation of the growth medium with Cr in monolayer cultures slightly increased proliferation and clearly stimulated metabolic activity, ALP activity and mineralization. This was in contrast to what was observed in micromass cultures, where Cr promoted ALP activity and mineralization, but not metabolic activity. The beneficial effects of Cr on ALP activity were more pronounced at the higher concentration of 20 mM Cr. However, this high concentration showed also some adverse effects on the viability of rat calvarial osteoblast-like cells at 3 weeks. This reduction in the viability might represent a general cell culture artefact and a dose response of the cells to Cr supplementation. As the cells proliferate, differentiate and form nodules, there is an increase in cell density, extracellular matrix and mineralization. This could lead to cell death as the diffusion of nutrients to the cells, which are localized near the culture dish surface and within the nodules, is reduced. These findings were supported by the ultrastructure of the cells in monlayers, where in most cases also some dead cells were observed within the nodules of all groups at 3 weeks. There was a dose response to the higher concentration of 20 mM Cr, which significantly reduced the viability of the cells as compared to control and 10 mM Cr group. This effect was less pronounced in monolayer cultures. Presumably, the presence of dead cells could lead to an increased mineralization (Boskey *et al.*, 1991).

Although 20 mM Cr reduced the viability of the cells, it exerted significant stimulatory effects on metabolic activity and differentiation. This is in accordance to other *in vitro* studies, where protective effects of 10 mM Cr against inhibition of mitochondrial respiration by methylglyoxal could be shown in cardiac cells (Roy *et al.*, 2003) and where 20 mM Cr improved intracellular Ca^{2+} handling and survival of mdx-mouse muscle cells (Pulido *et al.*, 1998). In addition, protection of Cr pre-treatment in ischemia of mammalian neurons was maximal with 10 mM Cr and no additional protection was provided by up to 20 mM Cr (Zapara *et al.*, 2004). On the other hand, the addition of up to 50 mM Cr had no influence in proliferation of Hodgkin disease-derived tumor cell lines (Kornacker *et al.*, 2001). Thus, if any negative effects by

high concentrations of Cr were exerted on cells in culture, such effects seem to strongly depend on the kind of cells that are used.

The importance of cellular energy for proper functioning of bone and cartilage cells is corroborated by the fact that interventions that interfere with ATP generation in these cells, either in culture or within intact bone, have dramatic effects, e.g. inhibition of glycolysis causes both a reduction in collagen synthesis (Ramp *et al.*, 1994) and a hypermineralization in tibiae of chick embryos (Ramp, 1975). For example, acute treatment of mature osteoblasts with metabolic inhibitors showed that the rate of glycolysis rose to maintain the cellular energy supply constant (Komarova *et al.*, 2000) and pyruvate deficiency in cultured calvarial osteoblasts during proliferation caused cell death (Hinoi *et al.*, 2002). On the other hand, inhibition of the activity of NAD-dependent enzymes associated with the production of ATP impairs cartilage formation resulting in limb shortening (Sheffield and Seegmiller, 1980). Furthermore, administration of b-guanidinopropionic acid (GPA; a Cr analogue and competitor of Cr uptake into the cell), which leads to a drastic reduction of intracellular PCr and Cr levels and thus interferes with the CK system, results in a disturbed enchondral bone formation *in vivo* and *in vitro* (Funanage *et al.*, 1992). These data, obtained by Cr-depletion with GPA, stress the importance of the CK/PCr system for normal bone and cartilage formation and physiological function. The fact that Cr has been shown to stimulate oxidative phosphorylation of isolated mitochondria that contain CK, thus increasing the net production of PCr, (Kay *et al.*, 2000), would indicate that increasing total Cr levels in a given cell leads to a generally increased availability of ATP, as shown for example in muscle cells (Wyss and Wallimann, 1992). In cartilage and bone, Cr may exert similar cell protective effects as described in muscle (Pulido *et al.*, 1998) and neuronal cells (Brewer and Wallimann, 2000) for review see (Wyss and Schulze, 2002) by improving the cellular energy state (higher PCr levels), and keeping the thermodynamic efficiency of ATP consuming processes, e.g. Ca^{2+} homeostasis high (high local ATP/ADP ratios) (Pulido *et al.*, 1998; Wallimann *et al.*, 1999; Passaquin *et al.*, 2002), by stimulating mitochondrial respiration (Kay *et al.*, 2000) and by protecting mitochondria from going into permeability transition and swelling (O'Gorman *et al.*, 1997; Dolder *et al.*, 2001; Dolder *et al.*, 2003; Speer *et al.*, 2005), that would eventually lead to cell death by apoptosis. As observed here, Cr supplemented osteoblast-like cells showed morphologically better preserved, less swollen mitochondria compared to controls without Cr. This finding is supported by the recent observations that the presence of mitochondrial CK together with Cr stabilized mitochondrial contact sites between inner and outer mitochondrial membranes, thus preventing swelling (Speer *et al.*, 2005). Thus, besides improving cellular energetics via an elevation of the PCr/ATP ratio, some effects of Cr observed here in osteoblast-like cells may additionally be related to protective effects of Cr on mitochondrial structure and function.

Precise coupling of spatially separated intracellular ATP-producing and ATP-consuming processes is fundamental to the bioenergetics of living organisms. The CK/PCr circuit ensures a fail-safe operation of these energetic systems over a broad range of cellular functional activities by stabilizing cellular ATP levels and keeping ATP/ADP ratio low (Dzeja and Terzic, 2003). As Cr supplementation increases intracellular PCr /ATP ratios, cellular energetics are additional stabilized (Wallimann *et al.*, 1992). Interestingly, Cr supplementation also increases glucose oxidation and phosphorylation of AMP-activated protein kinase (AMPK), and at the same time reduces lactate production in L6 rat skeletal muscle cells (Ceddia *et al.*, 2004). Thus, some effects of Cr on osteoblast-like cells could also be mediated by Cr affecting AMPK as an intracellular energy sensor that helps to maintain cellular energy balance by keeping ATP concentration stable within the cell (for review see Carling, 2004). Indeed, ATP availability and ATPase activity parallel the metabolic activity, viability and matrix synthesis of osteoblasts (Heyden and From, 1970; Tonna and Severson, 1971). This fact and the various mechanisms of action of Cr (see above) may explain the possible effects of Cr supplementations observed here.

In conclusion, micromass culture promotes the differentiation of primary osteoblast-like cells over monolayer cultures by decreasing proliferation and by increasing ALP activity and mineralization. The observed stimulatory effects of external supplementation with the energy precursor, Cr, were dependent on the organizational level of the cells in such a way that osteoblast-like cells in monolayer culture showed a significantly increased metabolic activity, ALP activity and mineralization in the presence of Cr in the growth medium. In micromass culture, Cr also significantly enhanced ALP activity and mineralization over the control group. The effect on ALP activity was more pronounced at the higher concentration of Cr, but 20 mM Cr had some adverse effects on the viability.

Based on this study and the literature, it is clear that cellular energetic is important for bone development, differentiation and maintenance and thus directly related to osteoporosis. Thus Cr supplementation could possibly used as adjuvant therapy for bone fracture healing or within a regime of osteoporosis treatment.

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Discussion with Reviewers

J. Gasser: The authors point at the importance of a good cell viability with examples from the literature. According to their work, creatine at 20 mM decreases cellular viability. Have the authors tested in their system whether creatine induces cell apoptosis (caspase activity for example)?

Authors: So far we have not done this yet, but we plan to do it in the near future. The ultrastructure of the cells is the only information we have at the present time addressing your question of whether apoptosis is taking place or not. There were, however, no indications of more dead cells being present in the 20 mM creatine group than in the 10 mM creatine group or in the control group. Under all conditions, dead cells were found at 3 weeks, which is in accordance with the literature. There is abundant literature concerning necrosis and apoptosis during bone formation

in vivo and *in vitro*. In this study, Cr treated cells showed better preserved mitochondria than the control group. One reason why 20 mM creatine in the medium under the conditions used, may have had a slight effect on osteoblast cell viability may be an osmotic effect, since creatine is taken up by a specific creatine transporter that co-transports sodium and chloride together with creatine (co transport), which could lead, if creatine is taken up fast enough, to a short-time hyper osmotic condition.

To address the question of an osmotic effect, we performed a dose response curve of Cr under iso-osmotic conditions using human osteoblast-like cells (HOB) *in vitro*. The concentrations of Cr were ranging from 1 to 20 mM. The osmolarity was adjusted to 20 mM with mannitol in all groups. The standard growth medium was used as a control for the effects of mannitol alone. The cells were kept in culture for 2 weeks. The viability and metabolic activity were analysed by neutral red (NR) uptake and MTT assay, respectively. NR uptake and the MTT reduction of the cells kept either in the standard growth medium or in the standard medium with 20 mM mannitol were similar. This shows that an increase in the osmotic pressure by the addition of 20 mM mannitol had no effects on the viability and metabolic activity of HOB. Cr supplementation decreased the neutral red uptake of HOB in a dose dependent manner, where the 10 mM Cr group was significantly lower than the control, and 20 mM Cr group was significantly lower than all tested concentrations of Cr and the standard growth medium alone. This was in contrast to the MTT reduction normalized to the neutral red uptake, which was significantly stimulated by the supplementation of the growth medium with 20 mM Cr. These results clearly indicate that the observed reduction in the neutral red uptake and the stimulation of the metabolic activity were due to the addition of Cr and not to an increase in osmotic pressure.

J. Caverzasio: Is it possible that increased metabolic activity in cells exposed to creatine accelerates cell apoptosis and necrosis and that this might contribute to the higher mineralization that the authors observed?
Authors: We analysed the metabolic activity by using the MTT assay. MTT enters the cells by endocytosis. Most cellular MTT reduction occurs outside of the mitochondrial inner membrane and involves NADH and NADPH-dependent mechanisms (Berridge and Tan, 1993). There is evidence that mitochondrial, cytosolic and microsomal enzymes reduce MTT (Gonzalez and Tarloff, 2001). Reduced MTT is then exocytosed and appears as needle-like crystals on the cell surface (Liu, 1999). A decrease in cellular MTT conversion does not necessarily mean cell death or cell growth inhibition, but can be influenced by factors, which have effects on endocytosis, cellular redox potential and exocytosis (Liu, 1999). In our study, Cr stimulated the MTT reduction, meaning that the cells supplemented with Cr could have a higher content of NAD(P)H than the control. Apoptosis of osteoblasts can be induced by NO as an example (Chen *et al.*, 2002; Chen *et al.*, 2005). The authors find, that NO release by sodium nitroprusside caused a time- and concentration-dependent decrease in MTT reduction and ALP activity and at the same time lead to an increase in the percentage of osteoblasts undergoing apoptosis by decreasing cellular ATP content, which leads to cell dysfunction. Similarly in myocytes, energy depletion induced by KCN and 2-deoxy-D-glucose, induced a decrease in the PCr / ATP ratio, as well as a MTT reduction concomitant with stimulated apoptosis (Engelbrecht *et al.*, 2004). Furthermore, MTT reduction accurately determines apoptotic death of neurons (Lobner, 2000). These findings indicate that ATP depletion is an important factor for induction of apoptosis. As pointed out in the discussion, Cr has clear protective effects in other cell types on mitochondrial structure and function, which is a key element in the induction of apoptosis. In addition, MTT reduction and ALP activity were positively affected by Cr supplementation in our study. Therefore, it is rather unlikely that Cr supplementation itself could lead to apoptosis.

R. Oreffo: The physiological relevance of creatine concentrations advocated and effects observed are unclear could you comment on the significance for the bone community?
Authors: Some details concerning the concentrations of creatine used by other laboratories in relation to the ones applied here are indicated in the discussion. As the reviewer can see, we are using the same concentration, which may seem high at first glance, but considering that for example muscle tissue contains up to 50mM of intracellular total creatine concentration, the concentration range used here may seem appropriate.

Since bone is a non-muscle tissue, which contain generally between 5 and 20 mM total creatine, the concentrations chosen in our experiments seem reasonable.

Bioenergetics may be a key element to understanding the changes observed in bone diseases such as osteoporosis. Deficient bone formation in metabolic bone diseases with a lower than normal osteoblastic population results from an impaired proliferative capacity of osteoblastic cells present along the trabecular bone surface (Marie and De Vernejoul, 1993). This could possibly be attributed to inhibition of glycolysis, which is the major energy source for immature osteoblasts (Komarova *et al.*, 2000, text reference). Progressive differentiation coincides with changes in cellular metabolism and mitochondrial activity, which are likely to play key roles in osteoblast function (Komarova *et al.*, 2000). Interestingly, osteoblasts derived from osteopenic rat bone show an increased respiration rate as compared to normal cells (Fini *et al.*, 2001, text reference).

Maintenance of adult skeletal mass is controlled not only by changes in the production of osteoclasts and osteoblasts but also by altering the duration of their respective lifespan through regulated apoptosis (Weinstein and Manolagas, 2000). Osteoblast apoptosis has been shown to be important in the pathogenesis of certain metabolic bone diseases such as glucocorticoid-induced osteoporosis, postmenopausal osteoporosis and involutional osteoporosis (for review see Manolagas, 2000; Weinstein and Manolagas, 2000; Weinstein, 2001; Jilka,

2003; Rehman and Lane, 2003). Furthermore, there could be a link between diabetes as major metabolic disease and bone formation as diabetic persons have a higher risk for osteoporosis than the average population (for review see Leidig-Bruckner and Ziegler, 2001; Chau *et al*., 2003).

Therapies that prevent or reverse osteoporosis act at least in part by preventing osteoblast apoptosis and/or by stimulating osteoclast apoptosis (Manolagas, 2000; Jilka, 2003). Thus, creatine may exert similar cell protective effects in bone as described in the discussion of the paper.

Additional References

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