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Extracellular pH Modulates the Activity of Cultured Human Osteoblasts

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Abstract The effect of medium pH on the activity of cultured human osteoblasts was investigated in this study. Osteoblasts derived from explants of human trabecular bone were grown to confluence and subcultured. The first-pass cells were incubated in Hepes-buffered media at initial pHs adjusted from 7.0 to 7.8. Osteoblast function was evaluated by measuring lactate production, alkaline phosphatase activity, proline hydroxylation, DNA content, and thymidine incorporation. Changes in medium pH were determined from media pHs recorded at the beginning and end of the final 48 h incubation period. As medium pH increased through pH 7.6, collagen synthesis, alkaline phosphatase activity, and thymidine incorporation increased. DNA content increased from pH 7.0 to 7.2, plateaued from pH 7.2 to 7.6, and increased again from pH 7.6 to 7.8. The changes in the medium pH were greatest at pHs 7.0 and 7.8, modest at pHs 7.4 and 7.6, and did not change at 7.2, suggesting that the pHs are migrating towards pH 7.2. Lactate production increased at pH 7.0 but remained constant from 7.2 to 7.8. These results suggest that in the pH range from 7.0–7.6 the activity of human osteoblasts increases with increasing pH, that this increase in activity does not require an increase in glycolytic activity, and that pH 7.2 may be the optimal pH for these cells. J. Cell. Biochem. 68:83–89, 1998.

Key words: pH; osteoblasts; collagen synthesis; alkaline phosphatase activity; glycolysis; DNA synthesis

Environmental factors such as blood flow, nutritional supply, and mechanical stress greatly influence the activity of osteoblasts, the bone-forming cells [Kita et al., 1987; Kubota, 1989; Neidlinger-Wilke et al., 1995; Reeve et al., 1988; Reolofsen et al., 1995; Stanford et al., 1995; Tuncay et al., 1994; Whiteside et al., 1977]. The changing conditions of the milieu surrounding osteoblasts may play important regulatory roles in these cells. Proliferation, differentiation, and phenotypic expression of osteoblasts may be affected by changes in their local environment.

Extracellular pH has been shown to modulate the activity of embryonic chick osteoblasts in vitro [Ramp et al., 1994]. Phenotypic expression (alkaline phosphatase, collagen synthesis) decreases with decreasing pH, while DNA content and synthesis remains constant. In the body, periods of low bone blood flow can occur in disuse, aging, and initial stages of fracture healing [Kita et al., 1987; Neidlinger-Wilke et al., 1995; Whiteside et al., 1977; Reeve et al., 1988], resulting in decreased ability to nourish bone cells and to remove metabolic waste products, such as lactic acid. The latter event is speculated to decrease the local pH surrounding osteoblasts, corresponding with the decreased activity of osteoblasts seen in studies of low blood flow [Reeve et al., 1988; Whiteside et al., 1977]. In addition, it has been shown that in metabolic acidosis the activity of the osteoblasts declines, but in metabolic alkalosis osteoblastic activity increases [Bushinsky, 1995, 1996; Ori et al., 1995; Sprague et al., 1994; Krieger et al., 1992]. Yamaguchi and coworkers [1995] have demonstrated that increases in extracellular pH increase the number of gap junctions formed between osteoblasts. Results from these studies strongly suggest that environmental pH may play a significant regulatory role in osteoblastic function. The synthesis, mitogenesis, and energy metabolism of human osteoblasts with respect to environmental pH have not been investigated. The purpose of this study was to determine if osteoblasts derived from explants of human trabecular bone are influenced by extracellular pH.

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MATERIALS AND METHODS

Cell Culture

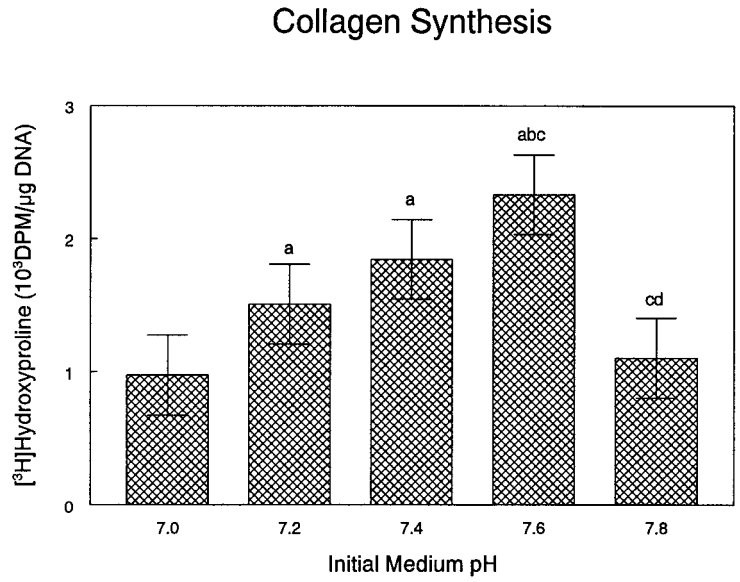
Osteoblasts were isolated from normal human trabecular bone from the iliac crest obtained at the time of surgery using a modification of a previously established method [Robey and Termine, 1985]. Trabecular bone fragments were minced and mechanically cleaned of loosely adherent tissue by repeatedly washing with medium (without fetal bovine serum [FBS]). The tissue was further cleaned by enzymatic digestion using collagenase (375 units/ml) (Type VII; Sigma Chemical Company, St Louis, MO). The cleaned and minced tissue was placed in 35 mm plates containing a modified phenol red– and Ca^{++}-free medium consisting of Dulbecco's modified Eagle's:Ham's F12 base medium (1:1 mixture; pH 7.4) (Sigma) with 20% FBS, 0.1% insulin-transferrin-selenium (Collaborative Biomedical Products, Bedford, MA), 40 ng/ml vitamin B_{12}, 2 µg/ml *p*-aminobenzoic acid, 200 ng/ml biotin, 35 µg/ml glycine, 250 mM glutamine, 50 mM lysine, 45 mM leucine, 2 mM methathionine, 8 mM $MgSO_4$, 100 U/ml–100 µg/ml–250 ng/ml penicillin-streptomycin-Fungizone (Sigma), and 100 µg/ml ascorbic acid. Cultures were incubated at 37°C in a humidified incubator and fed three times per week until the cells that migrated from the explants reached confluence. The cells were then subcultured after release with a protease E (7.5 units/ml) (Type VII; Sigma)/collagenase (375 units/ml) (Type VII; Sigma) solution, and the cells were divided, centrifuged, and resuspended in the medium described above at pH 7.0, 7.2, 7.4, 7.6, or 7.8. The buffer used in this study, Hepes, is a strong organic buffer between pHs 7.0 and 8.0. Human osteoblasts are unaffected when this buffer (15 mM) is used. Cells were plated at 2.5×10^4 cells/cm^2 in 35 mm, six-well plastic culture plates. At confluence, half of the wells at each pH were incubated with medium containing either 1 µCi/ml [^{3}H]proline (spec. act. 30.0 Ci/mmol) (Amersham Life Science, Inc., Arlington Heights, IL) and cultured for an additional 48 h or 2 µCi/ml [^{3}H]thymidine (spec. act. 84.0 Ci/mmol) (Amersham) and cultured for an additional 24 h. After this time, medium was removed and stored at −20°C until subsequent analyses. The cell layers were rinsed in saline and subjected to three cycles of freeze-thaw lysis in 1 ml of 1 M NaCl–0.1% Triton X-100–0.01% trypsin inhibitor (soybean Type II; Sigma). The cell layers were stored at −20°C until subsequent analyses. The human osteoblasts used in this study have been shown to contain high alkaline phosphatase activity, produce Type I collagen, increase cAMP content in response to parathyroid hormone challenge, and produce osteocalcin when incubated in the presence of 1,25 dihydroxycholecalciferol [Kaysinger et al., 1997].

Analytical Methods

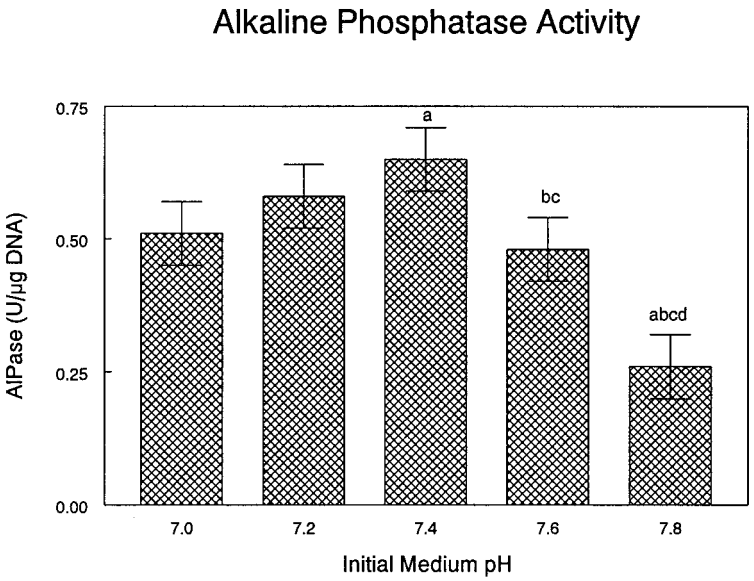
Lactate released to the medium, an index of glycolysis, was measured enzymatically [Lundholm et al., 1963]. The media were adjusted to the appropriate initial pHs using 0.1 N HCl and 0.1 N NaOH and a standard pH meter and electrode (model 245; Corning, Cambridge, MA). The pHs of the media incubated with and without cells were measured at the end of the incubation period. An aliquot from the cell layer lysate was removed for measurement of alkaline phosphatase activity, enzyme characteristic of osteoblasts. Cell layer alkaline phosphatase activity was determined at pH 9.8 using *p*-nitrophenylphosphate as the substrate [Lowry et al., 1957]. The enzymatic activity is expressed in units where 1 unit represents 1 µmol of substrate hydrolyzed per hour at 37°C. The cell layers were digested with Proteinase K (5 mg/ml) (Sigma) by shaking at 2 Hz overnight in a 60°C bath. An aliquot from the cell layer was removed for determination of DNA content, a measure of cell number. Total DNA content of the cell layers was determined fluorometrically using PicoGreen™ (Molecular Probes, Eugene, OR). The cell layers incubated with [^{3}H]proline were hydrolyzed overnight at 110°C. The HCl was evaporated with air using a sample dryer (Reacti-Therm; Pierce Chemical Co., Rockford, IL). The dried hydrolysates were resuspended

Fig. 1. Effect of medium pH on collagen synthesis (**A**), cellular alkaline phosphatase activity (**B**), and glycolysis (**C**) in human osteoblasts. Cells were grown in medium adjusted to the initial pHs shown. When confluent (7 days), cells were incubated in medium adjusted to the appropriate pH and containing 1 µCi/ml [^{3}H]proline for 48 h. Alkaline phosphatase activity and collagen synthesis were measured in the cell layers. Glycolysis was measured by lactate released to the medium. Means are normalized to DNA content, a measure of cell number. Each bar represents the mean of five wells. Error bars are the 95% confidence intervals. Data were analyzed by a one-way ANOVA followed by a Tukey-Kramer HSD multiple comparison test. [a]$P < 0.05$ compared with pH 7.0. [b]$P < 0.05$ compared with pH 7.2. [c]$P < 0.05$ compared with pH 7.4. [d]$P < 0.05$ compared with pH 7.6.

A


Collagen Synthesis
[3H]Hydroxyproline (10³DPM/µg DNA)
3
2
1
0
abc
a
a
cd
7.0
7.2
7.4
7.6
7.8
Initial Medium pH

B


Alkaline Phosphatase Activity
AlPase (U/µg DNA)
0.75
0.50
0.25
0.00
a
bc
abcd
7.0
7.2
7.4
7.6
7.8
Initial Medium pH

C

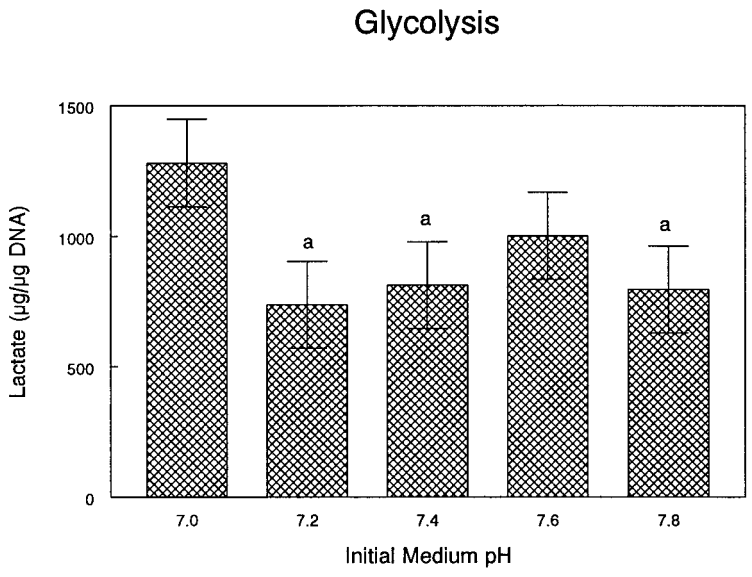

Glycolysis
Lactate (µg/µg DNA)
1500
1000
500
0
a
a
a
7.0
7.2
7.4
7.6
7.8
Initial Medium pH

Figure 1.

TABLE I. Initial and Final Medium pHS†

Initial medium pH	Final medium pH[a]	Change in pH
7.00	7.18 ± 0.01*	+0.18
7.20	7.22 ± 0.02	+0.02
7.40	7.34 ± 0.02*	−0.06
7.60	7.39 ± 0.01*	−0.21
7.80	7.39 ± 0.01*	−0.41

†Initial medium pH was the pH measured in the medium before addition to the cultures. Final medium pH was the pH measured in the media of five wells of cultured osteoblasts grown per pH.

[a]Values represent mean and 95% confidence intervals (n = 5).

*$P < 0.05$ compared to initial pH using Student's *t*-test. Before testing for significance, the pHs were converted to $[H^+]$.

in 200 mM NaOH (200 µl) and derivatized using 0.4 M $NaHCO_3$ (2 µl/µl hydrolysate) and dansyl chloride (9 mg/ml acetone, 6 µl/µl hydrolysate); 1 N acetic acid (1 µl/µl hydrolysate) was added to halt the reaction. The amino acids were separated by thin-layer chromatography and counted by liquid scintillation spectrometry to determine [^{3}H]hydroxyproline, a measure of newly synthesized collagen [Galvin et al., 1991]. The cell layers containing [^{3}H]thymidine were solubilized by the addition of NaOH and the protein and DNA precipitated using 10% trichloroacetic acid. The precipitates were collected on glass fiber filters and washed with 5% trichloroacetic acid. The incorporated [^{3}H]thymidine, an index of cell proliferation and DNA synthesis, was determined by liquid scintillation spectrometry [Centrella et al., 1988].

Statistical Analysis

A one-way analysis of variance followed by the Tukey-Kramer (HSD) multiple comparison analysis was performed on all the data, except for the comparison of initial and final medium pHs. For this comparison, the initial and final pHs were converted to hydrogen ion concentrations, and a Student's *t*-test was performed. Individual group standard deviations were used to construct the 95% confidence intervals. The data are presented as the mean (n = 5) and 95% confidence interval, with $P < 0.05$ considered significant.

RESULTS

As seen in avian osteoblasts [Ramp et al., 1994], biosynthetic activity of human osteoblasts increased with increasing pH. Collagen synthesis steadily increased and more than doubled from pH 7.0 to 7.6 (Fig. 1A); however, at pH 7.8, collagen synthesis decreased. Similarly, but not as profoundly, alkaline phosphatase activity increased 27% with increasing pH from 7.0 to 7.4 (Fig. 1B); however, the activity of the enzyme rapidly decreased above pH 7.4. Interestingly, this peaking of alkaline phosphatase activity with increasing pH was also shown in avian osteoblasts [Ramp et al., 1994].

Glycolysis was measured by the release of lactic acid into the incubation medium. With the exception of pH 7.0, lactate production was constant throughout the pH range (Fig. 1C). At pH 7.0, lactate production was dramatically increased 74% over pH 7.2, even though biosynthetic and mitogenic activities were decreased at this pH. Medium pH was measured at the beginning and end of the incubation periods (Table I). Media pH, when incubated for 48 h in the absence of cells, remained constant at all pHs. Even with Hepes as the buffer, the pHs of the cell media changed with time. The changes were largest at pHs 7.0 and 7.8 and modest at pH 7.4 and 7.6 and suggest that the media pHs migrated towards pH 7.2 when cells were present.

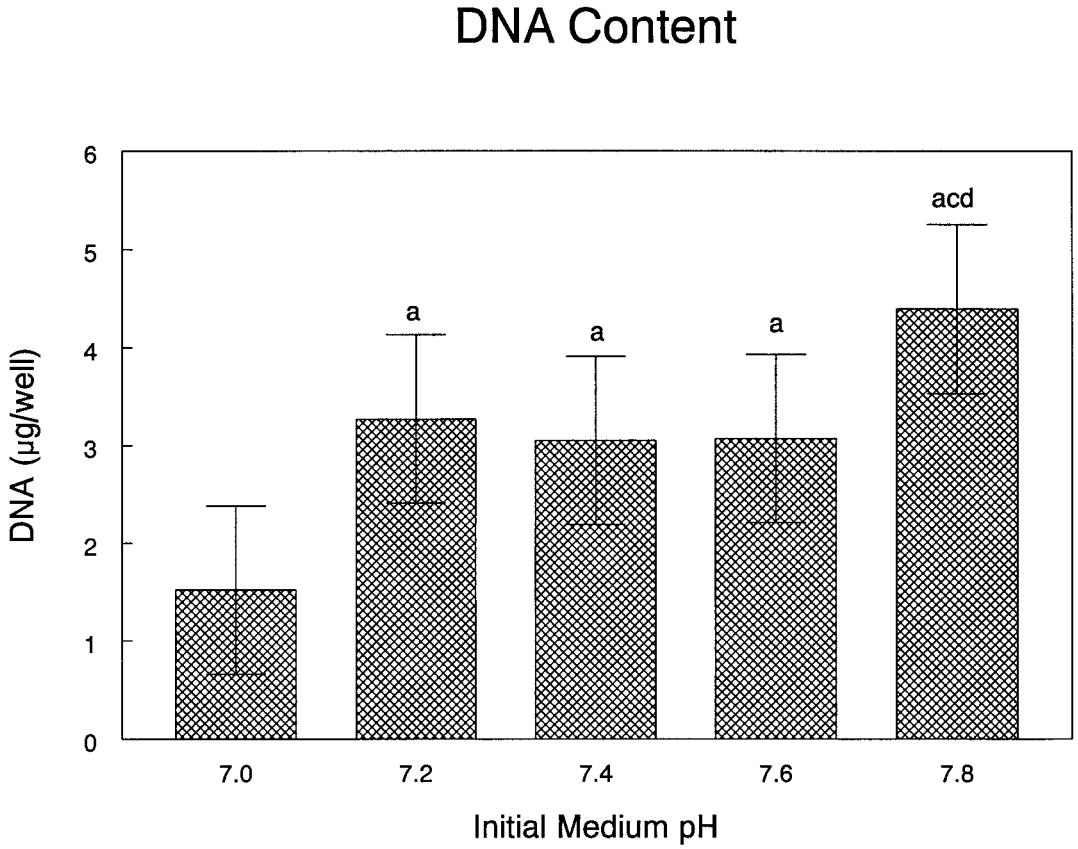
In terms of cell numbers, DNA content increased 115% from pH 7.0 to 7.2, plateaued between pHs 7.2 and 7.6, and increased 188% at pH 7.8 compared to pH 7.0 (Fig. 2A). DNA synthesis, which indicates rate of cell proliferation, increased 52% with increasing pH to 7.6 (Fig. 2B); however, at pH 7.8 DNA synthesis was not elevated compared to pH 7.0.

DISCUSSION

In this study, human osteoblasts have been shown to be sensitive to extracellular pH in vitro. Collagen synthesis and alkaline phosphatase activity, markers of osteoblastic activity,

Fig. 2. Effect of medium pH on DNA content (**A**) and DNA synthesis (**B**) in human osteoblasts. Cells were grown in medium adjusted to the initial pHs as shown. When confluent (7 days), cells were incubated in medium adjusted to the appropriate pH and containing 2 µCi/ml [^{3}H]thymidine for 24 h. DNA content and DNA synthesis were measured in confluent cell layers. For DNA synthesis, means are normalized to DNA content. DNA content is reported as "per well" data. Each bar represents the mean of five wells. Error bars are the 95% confidence intervals. Data were analyzed by a one-way ANOVA followed by a Tukey-Kramer HSD multiple comparison test. [a]$P < 0.05$ compared with pH 7.0. [b]$P < 0.05$ compared with pH 7.2. [c]$P < 0.05$ compared with pH 7.4. [d]$P < 0.05$ compared with pH 7.6.

A


DNA Content
DNA (µg/well)
0
1
2
3
4
5
6
a
a
a
acd
7.0
7.2
7.4
7.6
7.8
Initial Medium pH

B

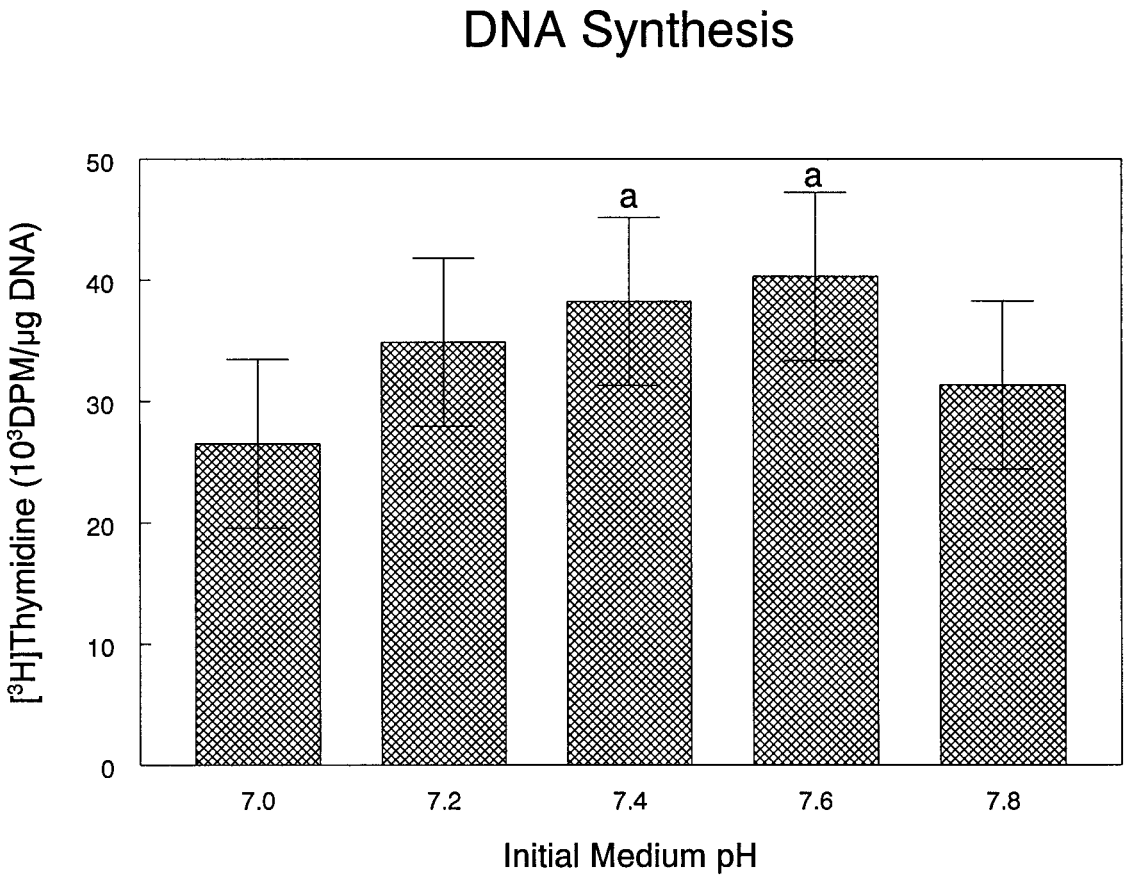

DNA Synthesis
[3H]Thymidine (10³DPM/µg DNA)
0
10
20
30
40
50
a
a
7.0
7.2
7.4
7.6
7.8
Initial Medium pH

Figure 2.

and cell proliferation increase with increasing pH; however, the pH range for these effects lies between pHs 7.0 and 7.6. At pH 7.8, these indices were decreased, which suggests that the optimal pH range for viable osteoblasts has been exceeded. At excessively high pHs (7.8 and higher), osteoblastic activity is markedly suppressed. This is similar but not identical to the results seen with avian osteoblasts cultured at similar pHs [Ramp et al., 1994]. Unlike the human osteoblasts, these avian cells were cultured for 48 h after reaching confluence at the various pHs. The activity of avian osteoblasts increased throughout the pH range tested (6.8–8.0), except for cell alkaline phosphatase, which peaked in activity in a fashion similar to the human osteoblasts. This comparison of avian to human osteoblasts suggests that human osteoblasts are more sensitive and less tolerant to alterations in extracellular pH than their avian counterparts. As noted above, DNA synthesis increased in a pattern markedly similar to collagen synthesis. Even though the study used mature osteoblast layers which downregulate proliferation once confluence has been reached, cell proliferation continues as a maintenance function of the cultures.

While the DNA content increases throughout the pH range tested, it does so in a more stepwise fashion, increasing from pH 7.0 to 7.2, remaining constant from 7.2 to 7.6, and increasing again at pH 7.8. With the exception of pH 7.8, these data agree well with the cell proliferation and biosynthesis data and suggest an increase in osteoblastic activity with increasing pH; however, decreased initial adherence of cells at pH 7.0 may also explain the decrease in DNA content in this group. The increase in DNA content without a concomitant increase in proliferation at pH 7.8 would suggest either that cells adhere more readily at the commencement of the incubation and/or that there is a decreased turnover of these cells. Whatever the reason for the increased cell number at pH 7.8, these cells demonstrate less osteoblastic phenotypic expression per cell than those at pH 7.6. This may indicate that a different phenotype or perhaps a different stage of osteoblastic development has been stimulated at this pH.

Because osteoblasts have a high rate of aerobic glycolysis and glycolysis is the major energy-producing pathway [Cohn, 1962], measuring the end product of glycolysis, lactic acid, is useful in determining general energy metabolism. Results from the measurement of lactic acid released to the medium suggest that at pH 7.2 and higher there is sufficient energy to meet any increased demands of the osteoblasts due to increased synthetic and mitogenic activities. Only at pH 7.0 was the lactate production increased, even though synthetic and mitogenic activities were decreased here. The need for this increased cellular energy is unknown. This is further complicated when the change in medium pHs is viewed globally; it appears that the osteoblasts are attempting to regulate their environmental pH and move it towards a more "physiologic" pH range of 7.2–7.4. This apparent ability of the osteoblasts to manipulate their environment from a sensed inappropriate one to a more acceptable environment is quite intriguing but not unique. Both astrocytes [Kaila et al., 1991] and colonocytes [Chu and Montrose, 1995] have also been shown to manipulate their extracellular pH.

It is known that in humans blood pH is tightly regulated, generally never fluctuating out of the 7.38–7.42 range [Laiken and Fanestil, 1985]. Only in special circumstances, such as metabolic acidosis, does the blood pH deviate from this physiological range. Although little is known regarding the pH of the bone interstitial fluid (BIF), it is likely that only in extraordinary circumstances does BIF pH dramatically fluctuate. This study indicates that small changes in BIF pH are enough to alter the activity of the human osteoblast. It is possible that in periods of low bone blood flow and poor oxygenation, such as with aging and the initial stages of fracture healing, the osteoblasts may experience these low pHs. Small shifts in extracellular pH over time could markedly affect the activity of osteoblasts. For example, in aging there is decreased bone blood flow which may result in insufficient removal of metabolic waste products and thereby decrease the extracellular pH near osteoblasts. Because of this decreased extracellular pH, the activity of the osteoblasts may be suppressed, and over time this could result in a measurable decrease in collagen and bone matrix synthesized, such as that seen in age-related osteoporosis.

It should be noted that this in vitro study does not necessarily mimic the in vivo human experience. Also, the human population is widely variable, and results from different patients may vary quantitatively.

In conclusion, this study has demonstrated a decrease in the biosynthetic and mitogenic activity of human osteoblasts with decreasing

extracellular pH. Although the pH of BIF bathing the osteoblasts is unknown, the pHs tested in this study are in the range possible for blood. This raises some intriguing questions regarding how osteoblasts interact with their surroundings and the importance of various microenvironmental factors in regulating the activity of these cells.

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