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An Optimized Method for the Chemiluminescent Detection of Alkaline Phosphatase Levels during Osteodifferentiation by Bone Morphogenetic Protein 2

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Abstract Differentiation of osteoprogenitor cells into osteoblasts is a pivotal step during the normal development and repair of bone. Upregulation of endogenous cellular alkaline phosphatase activity (AP) is a commonly used intracellular marker for the assessment of osteoprogenitor cell differentiation into the osteoblastic phenotype. Current methods for assaying AP involve colorimetric detection of the enzyme's activity using the synthetic substrate *p*-nitrophenol phosphate. In this paper, we explored an alternative method of detecting AP using the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) for enhanced AP sensitivity and a more simplified assay. Using calf intestinal alkaline phosphatase as a standardizing enzyme, we determined that the chemiluminescent detection system was four orders of magnitude more sensitive than the standard colorimetric method of detection. Moreover, the chemiluminescent assay was faster and markedly simpler to perform. To maximize the utility of this assay system, two osteoprogenitor cell lines were compared for their ability to generate alkaline phosphatases in vitro when exposed to recombinant human bone morphogenetic protein (rhBMP-2). The W20-17 cell line was substantially more sensitive to rhBMP-2 than the C3H10T1/2 cell line, where each cell line produced detectable increases in AP after exposure to rhBMP-2 levels of 5 and 25 ng/ml, respectively. The experimental design for AP responsiveness to rhBMP-2 was further optimized for chemiluminescent detection with the W20-17 cell line by comparing the effects of reporter cell seeding density and the day of assay. In summary, the data presented in this paper demonstrate a faster, simpler, and more sensitive chemiluminescent method to monitor changes in AP levels during osteodifferentiation. J. Cell. Biochem. 80:532–537, 2001.

Key words: osteoblast; alkaline phosphatase activity; osteoblastic differentiation; bone formation; BMP-2; osteoprogenitor cells; W20-17; C3H10T1/2

Differentiation of progenitor cells into mature cell types is fundamental to tissue formation and development. For bone formation, this process involves the differentiation of osteoprogenitor cells into a number of different phenotypes including osteoblast cells responsible for the synthesis and secretion of the bone matrix [Buckwalter et al., 1996]. While osteoblast differentiation is important during normal bone development, the biology of this process is also of considerable interest when applying therapies to enhance bone repair after injury or during disease processes [Balk et al., 1997; Caplan and Boyan, 1994; Ohgushi and Caplan, 1999].

To evaluate these osteoinductive therapies, an accurate and easy method is needed to quantify the progression into the osteoblastic phenotype both in vitro and in vivo. A wide range of markers exists that has been used for the identification of differentiation into the osteoblastic phenotype. Examples of such phenotype-specific markers include the production

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of bone matrix proteins osteopontin and osteocalcin, the process of mineralization, and the upregulation of the endogenous enzyme, alkaline phosphatase (AP) [Lian and Stein, 1992; Rodan and Rodan, 1983]. Quantification of AP activity has been routinely used to evaluate the osteoinductive capacity of both biomaterial surfaces and biological agents [El-Ghannam et al., 1997; Peter et al., 1998]. In particular, upregulation of AP has been a very useful marker to assess the ability of candidate bone therapeutics like bone morphogenetic protein 2 (BMP-2) to induce osteodifferentiation of progenitor cells in vitro [Lecanda et al., 1997; Takuwa et al., 1991; Wozney et al., 1988].

Current assays to measure endogenous AP activity use colorimetric detection methods, in which cellular AP cleaves the substrate *p*-nitrophenyl phosphate (NPP) to produce *p*-nitrophenol that can be measured by spectrophotometric assessment [Peter et al., 1998; Thies et al., 1992]. While the NPP substrate is useful, the chemiluminescent AP substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-phenyl phosphate (CSPD) provides the opportunity for a substantially higher level of sensitivity for AP detection and a somewhat simplified assay method over the current colorimetric methods to assess osteodifferentiation.

Given the promise of the chemiluminescent detection system, the objectives of this paper are: (1) to compare colorimetric versus chemiluminescent substrates for the detection of both purified AP and endogenous cellular AP in osteoprogenitor cells; and (2) to use the chemiluminescent assay to characterize experimental design parameters (i.e., assay evaluation time, cell seeding density, and osteoprogenitor cell line) for optimal recombinant human (rh)BMP-2 responsiveness in terms of AP activity.

MATERIALS AND METHODS

Materials

Cell culture. Dulbecco's Modified Eagles Medium (DMEM), L-glutamine, fetal bovine serum (FBS), and Gentamicin were purchased from Gibco (Grand Island, NY). W20-17 cell line and rhBMP-2 were generously given by Genetics Institute (Cambridge, MA).

Reagents. Calf intestinal alkaline phosphatase (CIAP) was purchased form Promega (Madison, WI). The *p*-nitrophenol phosphate (16 mM, NPP) was obtained from the ALP-50 alkaline phosphatase kit purchased from Sigma (St. Louis, MO). CSPD substrate (0.4 mM CSPD with Sapphire II, CD100RX) was purchased from Tropix (Bedford, MA). The bicinchoninic acid (BCA) kit was purchased from Pierce (Rockford, IL). Tris and Triton X-100 were purchased from Fisher Scientific (Fair Lawn, NJ).

Cell Culture

The mouse cell lines W20 clone 17 and C3H10T1/2 clone 8 were investigated in this study as they have both been reported to be responsive to rhBMP-2 in the literature [Katagiri et al., 1990; Thies et al., 1992]. These cells were cultured at a subconfluent density in DMEM supplemented with 8 μM L-glutamine, 10% heat inactivated FBS, and 0.5% Gentamicin. Cells were plated in 24-well dishes at the indicated cell density prior to assay. Seventeen to 24 h after plating the medium was replaced with a fresh medium containing varying concentrations of rhBMP-2. Cells were cultured for the appropriate time period and assayed for AP activity. If cells were cultured for 7 days, their medium was exchanged with a new medium containing the appropriate concentration of rhBMP-2 on day 3.

Alkaline Phosphatase Detection

CIAP was diluted several orders of magnitude from 1 to 10^{-7} units/ml in 25 mM Tris and 0.5% Triton X-100 pH ~ 8. For extraction of cellular AP, the medium was removed, cells were washed once with phosphate-buffered saline, and then lysed by three freeze–thaw cycles in 200 μl of 25 mM Tris and 0.5% Triton X-100 pH ~ 8.

For colorimetric AP detection, 20 μl of the AP sample were incubated with 1 ml of NPP substrate at 30°C for up to 5 min. The production of *p*-nitrophenol in the presence of AP was measured by monitoring light absorbance of the solution at 405 nm at 1 min increments on the spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Cambridge, UK). The slope of absorbance versus time plot was used to calculate AP activity. AP detection signal was recorded as change in absorbance per minute at 405 nm (ΔA/min).

For chemiluminescent AP detection, 20 μl of the AP sample were added to 100 μl of CSPD

substrate in a luminometer tube, vortexed, and incubated at room temperature for the indicated amount of time. The light output from each sample was integrated for 10 s after a 2 s delay by a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). AP detection signal was recorded in relative luminescence units (RLU).

Cellular AP activities were normalized to protein content with the BCA assay using bovine serum albumin to derive a standard curve. Samples were quantified in substrate signal (ΔA/min or RLU) or in CIAP activity units/ml calculated from the CIAP calibration curve. Data were presented as percent induction above unstimulated basal control cells as well as in units of AP from a CIAP standard curve.

Statistical Analysis

All data were taken in quadruplet and reported as mean and standard deviation. A paired Student's t-test of unequal variances with 95% confidence interval ($P < 0.05$) was done between the unstimulated basal control and each experimental condition. The lower threshold of rhBMP-2 responsiveness was determined to be the minimum concentration of rhBMP-2 to stimulate a statistically significant increase in AP activity over unstimulated basal control in osteoprogenitor cells.

RESULTS AND DISCUSSION

Calf intestinal alkaline phosphatase (CIAP) was used to compare the sensitivity of two detection methods: (1) using the colorimetric substrate, NPP; and (2) using the chemiluminescent substrate, CSPD. A standard curve over several logs of CIAP enzyme units was generated with NPP and CSPD (Fig. 1). CIAP detection extended over a linear range of signal on a logarithmic scale. Sensitivity of each detection method was determined as the lowest CIAP concentration on detection range to maintain the linear relationship (Table I). Similarly, the upper limit of detection was determined as the highest concentration of CIAP within the linear range (Table I). The lower detectable CIAP concentration limit for the CSPD incubated for 30 s (10^{-3} units/ml) showed a 10^2-fold higher sensitivity over NPP (10^{-1} units/ml). If the CSPD and sample incubation time is extended to 30 min (10^{-5} units/ml) the sensitivity increases 10^4-fold relative to NPP.

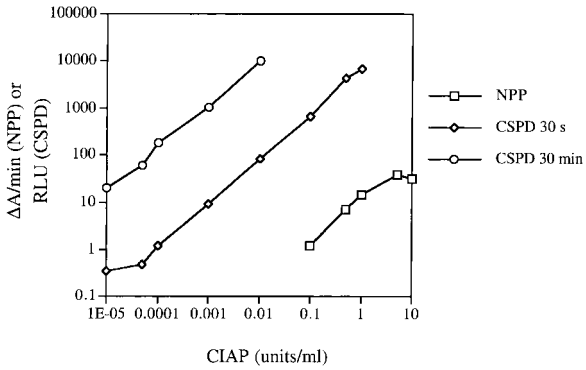

Fig. 1. Detection of calf intestinal alkaline phosphatase (CIAP) using the colorimetric substrate (NPP) and the chemiluminescent substrate (CSPD). The CSPD substrate was incubated for 30 s and 30 min. Data plotted in linear detection range on a logarithmic scale. AP was presented as substrate signal, change in absorbance per minute at 405 nm (ΔA/min) and relative luminescence unit (RLU) ($n = 4$).

Beyond issues of sensitivity and linear range, the experimental protocol for chemiluminescent detection of AP was less complicated and faster than the colorimetric detection method. For chemiluminescent detection, the assay can be performed at room temperature requiring a single reading per sample. By contrast, in the standard colorimetric detection method, the sample and substrate must be maintained at 30°C over the course of detection with multiple points required per sample.

We further compared the two detection methods in context of evaluating osteodifferentiation induced by an osteogenic agent. To accomplish this, we stimulated the osteoprogenitor cell line, W20-17, with rhBMP-2 and assayed for induced AP activity. W20-17 cells were exposed to rhBMP-2 (0–10 ng/ml), incubated for 2 days, and assayed for AP. Similar trends of endogenous AP activity for both detection methods were observed, when the substrate signal of stimulated cells was normalized

TABLE I.

Reagent	Lower detectable limit (units/ml)	Upper saturatable limit (units/ml)
p-Nitrophenol phosphate	$< 10^{-1}$	> 5
CSPD (30 s incubation)	$< 10^{-3}$	> 1
CSPD (30 min incubation)	$< 10^{-5}$	$> 10^{-2}$

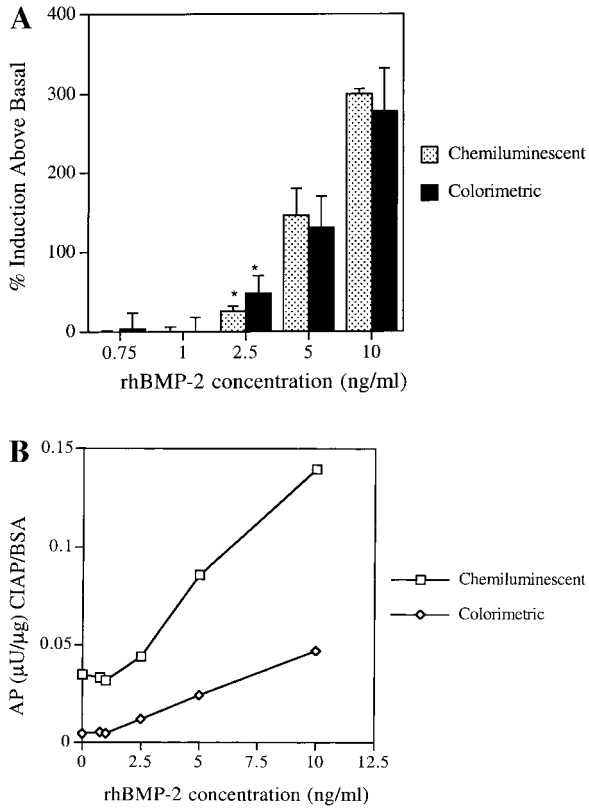

Fig. 2. Comparison of AP detection assays using cellular extracts of W20-17 cells harvested 2 days after exposure to rhBMP-2. Cells were plated at 60,000 cells per well. **A:** Data were expressed as percent induction of AP signal value above unstimulated basal control. The lower threshold of rhBMP-2 responsiveness was at 2.5 ng/ml for both detection methods ($^*P < 0.05$). Error bars represent means ± SD for $n = 4$. **B:** Data were interpolated from CIAP standard curve for respective detection method and normalized to protein content ($n = 4$).

to unstimulated control cells (Fig. 2A). The lower threshold of rhBMP-2 responsiveness, that is the minimum concentration of rhBMP-2 to stimulate a significant upregulation of AP over unstimulated control, was determined to be at 2.5 ng/ml rhBMP-2 for both assay substrates. Alternatively, we interpreted these data in terms of CIAP normalized to cellular protein content (Fig. 2B). Both detection methods followed similar trends; however, it was evident that chemiluminescent detection had an amplified level of AP signal relative to colorimetric detection. This amplified signal may prove useful when assessing cell populations with low levels of endogenous AP. While the detection of AP with the chemiluminescent method gains no major advantage over the colorimetric method for the detection of endogenous AP in response to rhBMP-2, the wider dynamic range, and the ease and simplicity of this assay warranted further inquiry.

To maximize the ability of the chemiluminescent system to detect rhBMP-2, a number of experiments were performed to optimize reporter cell line and the culture conditions of these cells. In the osteodifferentiation cascade, upregulation of AP is a transient process [Lian and Stein, 1992]. The time point of AP evaluation may be a critical experimental design parameter for identifying maximal AP responsiveness induced by an osteogenic stimulus. W20-17 cells were harvested and assayed for AP activity at 1, 3, and 7 days after initial stimulation with rhBMP-2 (0–100 ng/ml) (Fig. 3). All time points had a low threshold of rhBMP-2 responsiveness at 5 ng/ml. Incubation of rhBMP-2 on cells for 3 days or longer showed higher levels of AP induction than the 1-day time point.

Previous work in primary osteoblasts has shown that expression of osteoblastic markers such as AP is not initiated until after the proliferative period in the differentiation cascade [Owen et al., 1990]. To determine if cell proliferation affected rhBMP-2 responsiveness in the W20-17 cell line, we evaluated seeding densities representative of subconfluent, confluent, and overconfluent at the time of stimu-

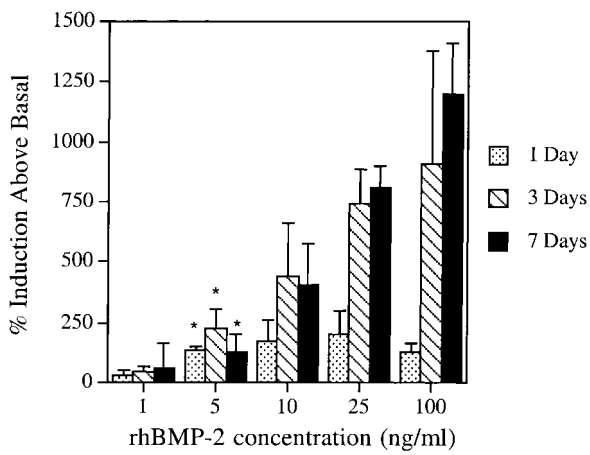

Fig. 3. AP upregulation stimulated by rhBMP-2 in W20-17 cells was investigated as a function of evaluation time point. Cells were seeded at 120,000 cells/well and assayed for AP at 1, 3, and 7 days after stimulation with rhBMP-2. Data were expressed as percent induction of AP per cellular protein content above unstimulated basal control. The lower threshold of rhBMP-2 responsiveness was at 5 ng/ml for both all time points ($^*P < 0.05$). Error bars represent means ± SD for $n = 4$.

lation. In a 24-well plate W20-17 cells were seeded at subconfluent (30,000 cells/well), confluent (60,000 cells/well), and overconfluent (120,000 cells/well). The seeding density of W20-17 cells did not significantly alter the lower threshold of rhBMP-2 responsiveness (5 ng/ml rhBMP-2) nor did it affect the amount of induction above basal for the lower concentrations of rhBMP-2 (Fig. 4). The relative independence of W20-17 cell seeding density and time of assay on rhBMP-2 responsiveness for AP upregulation suggest that this clonal cell line has differentiated past the proliferative period of the differentiation cascade.

Extensive research has investigated the effects of rhBMP-2 on many cell types ranging from primary cells to cell lines [Katagiri et al., 1990; Lecanda et al., 1997; Takuwa et al., 1991; Thies et al., 1992]. We investigated two representative rhBMP-2 responsive osteoprogenitor cell lines, W20-17 and C3H10T1/2, to identify the best reporter cell line to use in detecting rhBMP-2 bioactivity in vitro. When both cell lines were compared under optimal conditions, the W20-17 cells were five-fold more sensitive to lower concentrations of rhBMP-2 than C3H10T1/2 (Fig. 5). The lower threshold of rhBMP-2 responsiveness for W20-17 cells was 5 ng/ml; in contrast, the lower limit of rhBMP-2 responsiveness for C3H10T1/2 cells was 25 ng/ml. The lower threshold of rhBMP-2 responsiveness for C3H10T1/2 was not affected by time of evaluation or cell seeding density (data not shown). The relative level of differentiation in terms of endogenous AP may explain this difference in sensitivity between these cell lines. Results showed that the W20-17 cells had a significantly higher constitutive level of AP (expressed in units of CIAP per intracellular protein content) compared to the C3H10T1/2 cells (Table II).

In summary, chemiluminescent detection of AP provides a viable alternative to the current colorimetric detection methods for quantifying osteodifferentiation by BMPs and other osteogenic agents. This technique will likely prove useful for evaluating the potency of different osteoinductive gene delivery vectors and for assessing the function of recombinantly modified osteogenic proteins in vitro prior to testing in vitro. In addition, chemiluminescent detec-

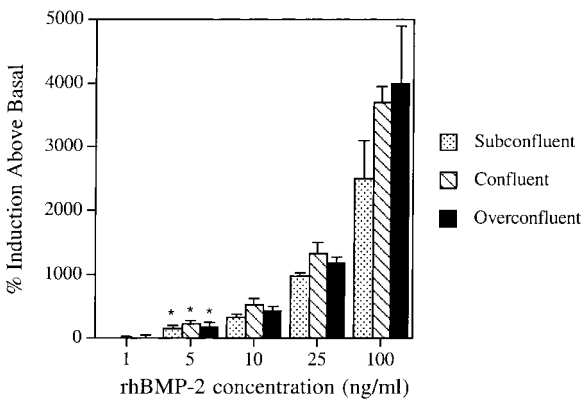

Fig. 4. AP upregulation stimulated by rhBMP-2 in W20-17 cells was investigated as a function of cell confluency. Cells were seeded at subconfluent (30,000 cells/well), confluent (60,000 cells/well), and overconfluent (120,000 cells/well) cell densities and assayed for AP at 3 days after stimulation with rhBMP-2. Data were expressed as percent induction of AP per cellular protein content above unstimulated basal control. The lower threshold of rhBMP-2 responsiveness was at 5 ng/ml for all cell densities ($*P < 0.05$). Error bars represent means ± SD for $n = 4$.

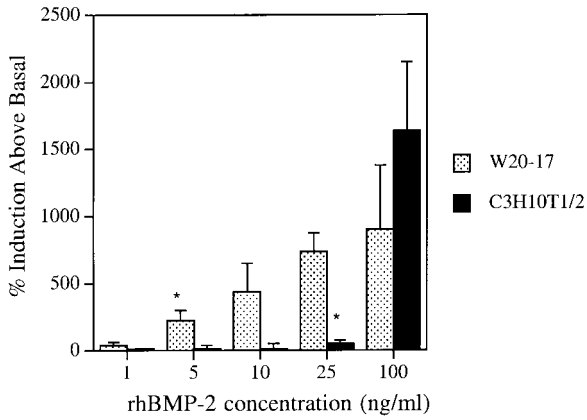

Fig. 5. AP upregulation stimulated by rhBMP-2 in osteoprogenitor cells was investigated as a function cell line. W20-17 and C3H10T1/2 cells were seeded at 120,000 cells/well and assayed for AP at 3 days after stimulation with rhBMP-2. Data were expressed as percent induction of AP per cellular protein content above unstimulated basal control. The lower threshold of rhBMP-2 responsiveness was at 5 ng/ml for W20-17 and 25 ng/ml for C3H10T1/2 ($*P < 0.05$). Error bars represent means ± SD for $n = 4$.

TABLE II.

rhBMP-2 (ng/ml)	W20-17 AP (μU/μg)	C3H10T1/2 AP (μU/μg)
0	336 (±124)	0.70 (±0.06)
25	2827 (±479)	1.10 (±0.16)
100	3386 (±1573)	12.29 (±3.61)

tion of AP may also provide a powerful strategy in assessing osteodifferentiation on 3-D porous materials for bone regeneration in critical sized defects. Extraction of cellular AP from these materials often results in polymer particulate contamination. When detecting AP colorimetrically, the inherent absorbance of these polymer contaminants in the sample can result in an elevated level of background signal, which makes it more difficult to assess absorbance changes in a sample over time. Since chemiluminescent detection of AP relies on emittance of light, the polymer contaminants will not increase background or interfere with detection. In conclusion, the chemiluminescent detection method of AP described in this paper should provide a useful tool for evaluation of a variety of osteoinductive therapies.

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