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Analytical Biochemistry 329 (2004) 77–84
ANALYTICAL BIOCHEMISTRY
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An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction

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Received 30 October 2003
Available online 20 April 2004

Abstract

Alizarin red S (ARS) staining has been used for decades to evaluate calcium-rich deposits by cells in culture. It is particularly versatile in that the dye can be extracted from the stained monolayer and assayed. This study describes a sensitive method for the recovery and semiquantification of ARS in a stained monolayer by acetic acid extraction and neutralization with ammonium hydroxide followed by colorimetric detection at 405 nm. This method was three times more sensitive than an older method involving cetylpyridinium chloride (CPC) extraction and resulted in a better signal to noise ratio, especially for weakly stained monolayers. The assay facilitates detailed inspection of mineralization by phase microscopy and semiquantification of the entire monolayer by extraction and quantification. The sensitivity of the assay is improved by the extraction of the calcified mineral at low pH and, since the mineral is already stained in a quantitative manner, there is no requirement for an additional colorimetric quantification step. Furthermore, the linear range is much wider than those of conventional assays for calcium, making dilutions of mineral extracts prior to measurement unnecessary. It has a wide range of potential uses including tumor characterization, mesenchymal stem cell evaluation, and osteogenic compound screening. Although more labor intensive than CPC extraction, the protocol is more sensitive and yields more reliable results for weakly mineralizing samples.

Endochondral ossification is the mechanism by which the majority of bones develop in higher vertebrates where the cartilaginous model of the skeleton is replaced by bone followed by subsequent longitudinal growth [1,2]. Calcification occurs at nucleation sites known as matrix vesicles present in the lacunae of mineralizing cartilage. They are believed to accumulate Ca^{2+} and inorganic phosphate which serve as nucleating agents for the formation of hydroxyapatite ($Ca_{10}(PO_4)6(OH_2)_2$), the main inorganic component of bone [3–5].

Ex vivo, some cell lines can be induced to form mineral in culture by treatment with osteogenic medium. Osteogenic medium contains a source of phosphate, ascorbic acid, and dexamethasone in a rich medium such as α-MEM containing fetal calf serum (FCS)[1] [6]. Mineralization can be assessed by a number of means including fluorescent calcein binding [7], Von Kossa staining [8], and Alizarin red S (ARS) incorporation [9–11]. Both Von Kossa and ARS staining allow simultaneous evaluation of mineral distribution and inspection of fine structures by phase contrast microscopy. ARS staining is particularly versatile in that the dye can be extracted from the stained monolayer and readily assayed. An alternative to assaying calcium levels would be to assay inorganic phosphate deposition but this approach may be problematic due to the high levels of contaminating phosphate associated with other components of the cell and the high levels of free phosphate in the cytosol. This study describes a sensitive method for the recovery and semiquantification of ARS in a stained monolayer based on acetic acid extraction and neutralization with ammonium hydroxide followed by colorimetric detection at 405 nm. The assay, more sensitive than CPC extraction [11], reduces variability and yields more reliable results for weakly mineralizing samples. Furthermore, the method boasts a much wider linear detection range than traditional calcium detection methods [11]. The mineralization

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[1] *Abbreviations used:* FCS, fetal calf serum; ARS, Alizarin red S; CPC, cetylpyridinium chloride; PBS, phosphate-buffered saline.

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doi:10.1016/j.ab.2004.02.002

assay described here is much more convenient than established calcium assays that have very limited linear detection ranges and do not allow direct visualization of mineral prior to processing and measurement. This assay has a wide range of potential uses including tumor characterization, mesenchymal stem cell evaluation, and osteogenic compound screening.

Materials and methods

Spectrophotometry

All reagents were purchased from Sigma–Aldrich (St. Louis, MO). ARS (40 mM) was prepared in dH_2O and the pH was adjusted to 4.1 using 10% (v/v) ammonium hydroxide. Scanning spectrophotometry was achieved using a dual-beam instrument at 25 °C (Beckman Coulter DU 640) and 1-cm-path-length acrylic cuvettes (Fisher Lifesciences). A 96-well plate reader (Benchmark Model, Bio-Rad Laboratories, Hercules, CA) was employed to measure absorbance during the assay. Duplicate plating demonstrated that error caused by pipetting and debris contributed to less than 10% variability. ARS solutions in CPC (10% w/v) were prepared in 10 mM Na_2PO_4 (pH 7.0) and scanned as above.

Tissue culture

hMSCs were prepared from bone marrow aspirates and cultured as previously described [6,12,13]. Cells were recovered by incubation with 0.25% (w/v) trypsin and 1 mM EDTA (Invitrogen, Carlsbad, CA) for 5–7 min at 37 °C, replated at 5000 cells per cm^2 in 10-cm^2 wells of six-well plates (Nunc, Fisher Lifesciences), and cultured until confluent. JEG3 epithelial carcinoma, HT1080 hepatocyte fibrosarcoma, and MG63 osteogenic sarcoma cell lines were acquired from the American Type Culture Collection (Rockville, MD) and cultured in accordance with their protocols. Cells were grown to confluency prior to addition of mineralizing medium. For the extraction and expansion of LS1 cells, a 2-g tissue biopsy from an osteoblastic tumor was recovered from a 12-year-old female during surgery. The tissue was digested using collagenase/dispase (Roche Diagnostics, Indianapolis, IN), recovered by centrifugation at 500 g for 15 min, washed twice in PBS, and added to a 150-cm^2 tissue culture dish. The resultant adherent cells were cultured as hMSCs.

Mineralization

Mineralization was induced on confluent monolayers by addition of α-MEM containing 20% (v/v) FCS, 100 μg mL^{-1} streptomycin, 100 U mL^{-1} penicillin, and 2 mM glutamine with osteogenic supplements, 1 mM sodium glycerophosphate, 50 μg mM L-ascorbate, and, in most cases, 10^{-8} M dexamethasone (all Sigma–Aldrich). Cultures were incubated at 37 °C with 5% CO_2 with changes of medium every 4 days.

Detection and quantification of mineralization

Monolayers in 6-well plates (10 cm^2/well) were washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma–Aldrich) at room temperature for 15 min. The monolayers were then washed twice with excess dH_2O prior to addition of 1 mL of 40 mM ARS (pH 4.1) per well. The plates were incubated at room temperature for 20 min with gentle shaking. After aspiration of the unincorporated dye, the wells were washed four times with 4 mL dH_2O while shaking for 5 min. The plates were then left at an angle for 2 min to facilitate removal of excess water, reaspirated, and then stored at −20 °C prior to dye extraction. Stained monolayers were visualized by phase microscopy using an inverted microscope (Nikon). For quantification of staining, 800 μL 10% (v/v) acetic acid was added to each well, and the plate was incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped from the plate with a cell scraper (Fisher Lifesciences) and transferred with 10% (v/v) acetic acid to a 1.5-mL microcentrifuge tube with a wide-mouth pipette. After vortexing for 30 s, the slurry was overlaid with 500 μL mineral oil (Sigma–Aldrich), heated to exactly 85 °C for 10 min, and transferred to ice for 5 min. Care was taken at this point to avoid opening of the tubes until fully cooled. The slurry was then centrifuged at 20,000*g* for 15 min and 500 μL of the supernatant was removed to a new 1.5-mL microcentrifuge tube. Then 200 μL of 10% (v/v) ammonium hydroxide was added to neutralize the acid. In some cases, the pH was measured at this point to ensure that it was between 4.1 and 4.5. Aliquots (150 μL) of the supernatant were read in triplicate at 405 nm in 96-well format using opaque-walled, transparent-bottomed plates (Fisher Lifesciences). Statistical analyses on three to six readings were carried out using Student's *t* test, and *p* values of less than 0.05 were considered significant. For CPC extractions, an adaptation of the protocol described in Stanford et al. [11] was followed. Monolayers on 6-well plates were washed five times in PBS and then fixed with ice-cold 70% ethanol for 1 h. The monolayers were then washed three times with dH_2O. ARS was extracted from the monolayer by incubation of the monolayers in 1 mL CPC buffer for 1 h. The dye was then removed and 200-μL aliquots were transferred to a 96-well plate prior to reading at 550 nm.

Arsenazo III calcium assay

To prevent contamination, all solutions were prepared using ultrapure reagents (Sigma), HPLC-grade

dH_2O, and sterile plasticware. Unfixed and unstained osteogenic monolayers of hMSCs were processed by acid extraction and neutralization as described above. In a 96-well microtiter plate, 10 μL of the extract was added to 100 μM Arsenazo III in 90 μL of dH_2O. After 10 min at room temperature, the absorbance of the samples was measured at 595 nm. Measurements were carried out in triplicate and for most of the samples, dilutions ranging from 10 to 0.3% in neutralized extraction buffer were necessary to allow measurement within the linear range of the assay.

Results

Spectrophotometric properties of ARS

Scans between 300 and 650 nm were carried out on the ARS diluted in ammonium acetate at numerous pH values between 3.5 and 6.0. The absorbance at 405 nm had greatest amplitude at pH 4.0–4.5 (Fig. 1A) and, over this range, fluctuations in pH had no detectable effect on the absorbance. Plots of absorbance versus ARS concentration demonstrated that as little as 40 μM dye in samples of 200 μL could be detected at 405 nm using a 96-well format assay. When diluted in 10% (w/v) CPC in 10 mM Na_2PO_4 (pH 7.0), the ARS exhibited an absorption maximum in the visible range between 545 and 560 (Fig. 1B) which was weaker in amplitude than the maximum detected for ARS at 405 nm in ammonium acetate at pH 4.1. An intense peak at approximately 349 nm was also detectable but since this peak is in the ultraviolet range it is likely to be affected by absorbing and fluorescing factors in the extracts themselves, causing inaccuracy. Indeed, extracts prepared in the absence of ARS exhibited relatively high and variable absorbances between 340 and 350 nm (data not shown), rendering it impossible to predict the level of background for each extract in the presence of ARS.

In ammonium acetate (pH 4.1), the relationship between ARS concentration and signal was linear between less than 50 μM and 2 mM (Fig. 2A) with an extinction coefficient of 1100 $M^{-1} cm^{-1}$, whereas absorbance of ARS in CPC exhibited a linear response between 250 μM and 2 mM with an extinction coefficient of 165 $M^{-1} cm^{-1}$.

Quantification of mineralization in differentiating hMSCs and MG63 cells

To develop an acid extraction protocol for the extraction and quantification of ARS, confluent monolayers of MG63 cells were prepared in 175-cm^2 flasks and cultured for 3 weeks in the dexamethasone-free osteogenic medium. On staining of the monolayers with ARS, a deeply stained mineralized layer was evident. The relationship between dye extraction and tissue input was investigated by recovering the monolayer and extracting the dye from various amounts of tissue. The mineralizing tissue was recovered by scraping, divided into specific

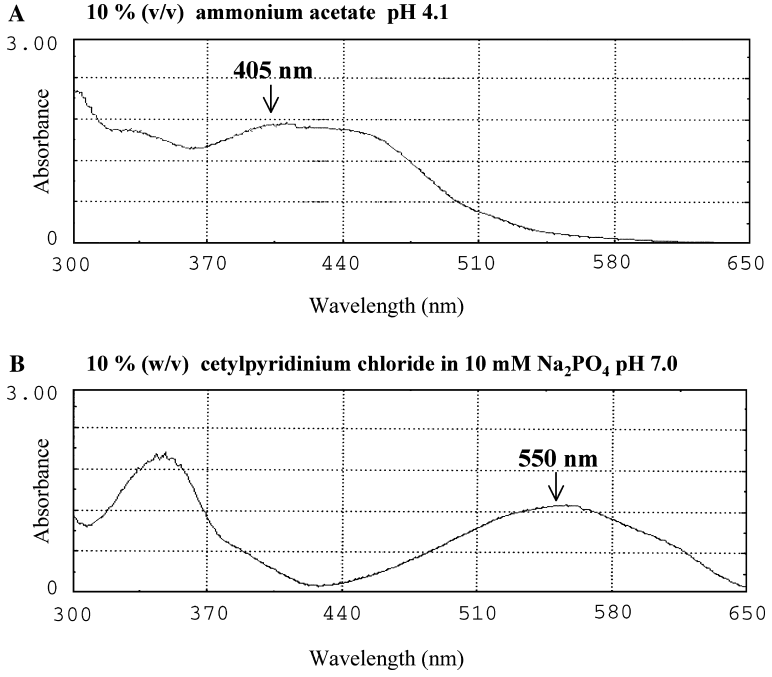

Fig. 1. (A) Scanning spectrometry of a solution of 40 mM ARS adjusted to pH 4.1 by titration with 10% (v/v) acetic acid and 10% (v/v) ammonium hydroxide. The peak at 405 nm is indicated. (B) Scanning spectrometry of a solution of ARS adjusted to pH 7.0 with Na_2PO_4 and 10% (w/v) cetylpyridinium chloride. The peak at 550 nm is indicated. In both cases, the buffers containing no ARS did not give a detectable absorbance over all wavelengths tested (data not shown).

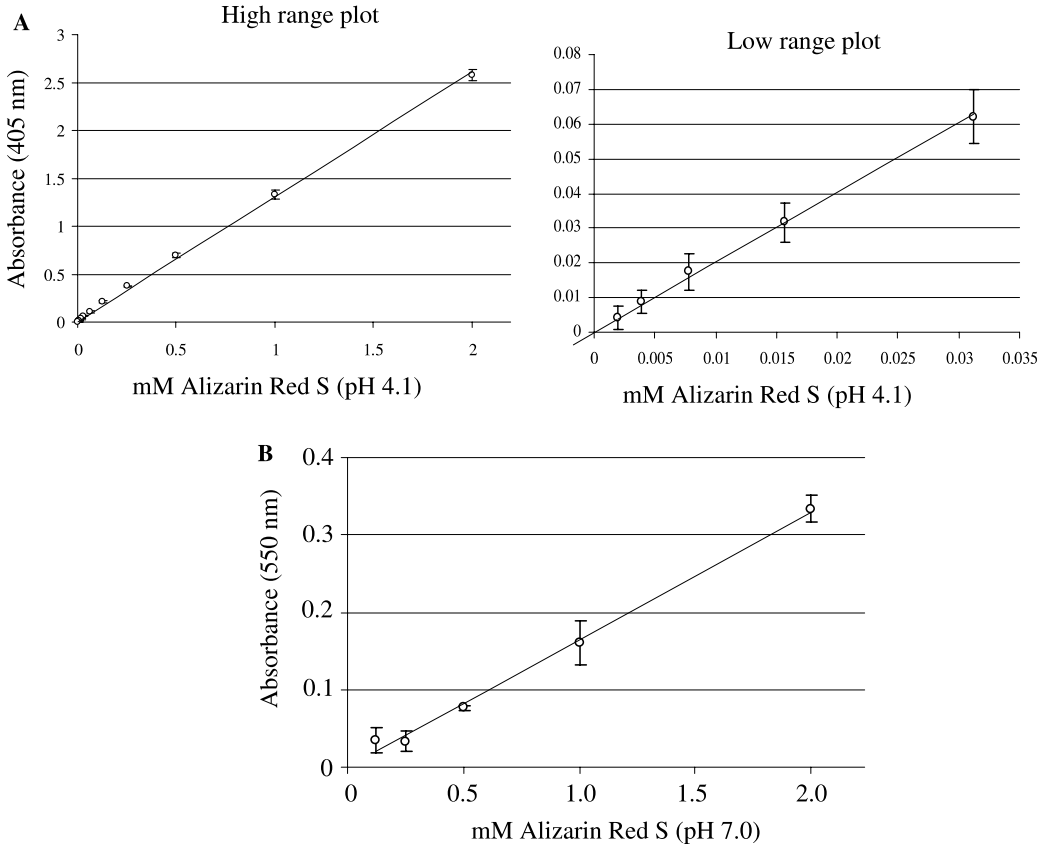

Fig. 2. (A) Plot of ARS concentration versus absorbance at 405 nm diluted with 10% (v/v) acetic acid and 10% (v/v) ammonium hydroxide. The plot remains linear between less than 50 μM ARS and 2 mM ARS. Data are expressed as the mean ($n = 5$) with error bars representing ±1 SD. (B) Plot of ARS concentration versus absorbance at 550 nm diluted with 10 mM Na_2PO_4 (pH 7.0) containing 10% (w/v) celylpyridinium chloride. The plot remains linear between 0.25 and 2 mM ARS but becomes unreliable below 0.25 mM. Data are expressed as the mean ($n = 3$) with error bars representing ±1 SD.

amounts by wet weight, and solubilized by heating at 85 °C in 10% (v/v) acetic acid for 10 min. Evaporation of the acid solution was prevented by overlaying the sample with excess light mineral oil. After heating, centrifugation, and neutralization, the absorbances (405 nm) of the clear red extracts were measured. A linear correlation between dye recovery and tissue wet weight confirmed that the efficiency of the extraction was unaffected by tissue mass between 0.2 and 15 mg (Fig. 3A). Both the acid extraction and the CPC assays were tested on mineralizing hMSCs. Confluent mineralizing cultures were established in six well plates over 30 days. At 10-days intervals, the cultures were stained with ARS, washed, and frozen at −20 °C. After 30 days, three wells from each interval were assayed by both methods. When compared with a room-temperature acid extraction, the 85 °C incubation significantly improved the dye extraction and hence contributed to the sensitivity of the assay (Fig. 3B). Although the acid extraction and CPC assays detected a rapid accumulation of mineral between day 10 and day 20 of incubation (Fig. 3C), the acid extraction assay was less variable and especially accurate in detecting low levels of mineral incorporation with statistical significance between readings at day 0 and 10 (Fig. 3C, inset). Although there was a minimal basal level of ARS staining at day 0, typical of dense cultures of hMSCs, CPC extraction failed to detect mineral accumulation before day 10. Combination of CPC and acidic conditions did not significantly improve the extraction efficiency compared with the use of acetic acid alone (data not shown).

To correlate microscopy with quantification, hMSCs from two donors were induced to mineralize in culture. Over 3 weeks, hMSCs from both donors gradually produced dye-stained mineral appearing as dense nucleation points that expanded over the entire monolayer after 16 days (Fig. 4A). Semiquantitative analysis of ARS staining revealed that the hMSCs treated with osteogenic substrate and dexamethasone produced significantly more stained mineral than untreated cells (Fig. 4B). Variation between replicated cultures was minimal, with standard deviations ($n = 6$) rarely exceeding 15% of the mean.

Arsenazo III for calcium quantification in mineralizing monolayers

We tested the suitability of Arsenazo III reagent [14,15] for assaying calcium derived from acid-extracted

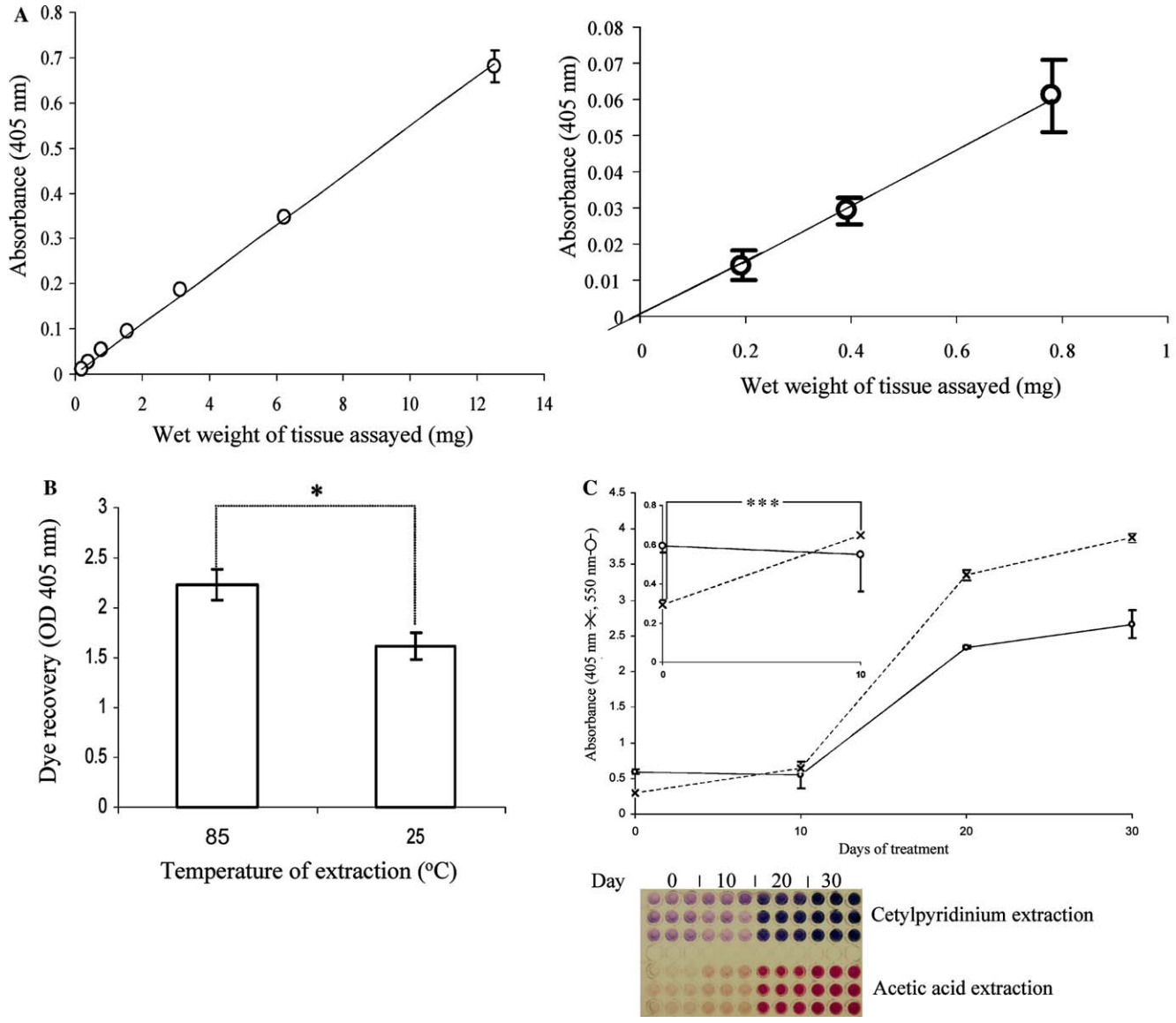

Fig. 3. (A) Linear relationship between ARS recovery and wet weight of tissue extracted between a range of 0.2 and 15 mg. (B) Comparison of the effects of incubation at 85 °C versus incubation at 25 °C during the acid extraction for osteogenic hMSCs after 3 weeks. Data are expressed as the mean ($n = 6$) with error bars representing ±1 SD. The p value for the data is < 0.005. (C) Comparison of cetylpyridinium chloride extraction with acid extraction. Confluent cultures of hMSCs were incubated in mineralization buffer for up to 30 days. At 10-day intervals, three cultures were stained with ARS and extracted by both techniques. The recovery with regard to both OD at 405 nm in the case of acid extraction (crosses) and OD at 550 nm in the case of cetylpyridinium chloride extraction (circles) is plotted. The inset details the readings taken at day 0 and day 10. Data are expressed as the mean ($n = 3$) with error bars representing ±1 SD. The three asterisks in the inset represent statistical significance of the acid extraction readings between day 0 and 10 readings with $p < 0.001$. A photograph of a microtiter plate containing cetylpyridinium and acid extracted ARS is presented below.

osteogenic MSCs. The linear range of the assay was reproducible between 0 and 80 μM (Fig. 5A). When compared with acid extraction of ARS, the Arsenazo III assay produced equivalent data but, due to the low and narrow linear range of the Arsenazo III assay, extensive dilution of the samples resulted in a high level of variation (Fig. 5B). Fixation of the samples did not appear to affect sensitivity or reproducibility since the acid extraction results were comparable to the Arsenazo III measurements that were carried out on unfixed hMSCs.

Quantification of mineralization in various established cell lines

JEG3, an epithelial choriocarcinoma cell line, HT1080, an hepatocarcinoma cell line, and LS1, a previously uncharacterized cell line established from an osteosarcoma biopsy taken from a 12-year-old female, were grown to confluency and incubated in osteogenic medium for 4 weeks. The assay was employed to follow mineralization of the cell lines. As expected, JEG3 and

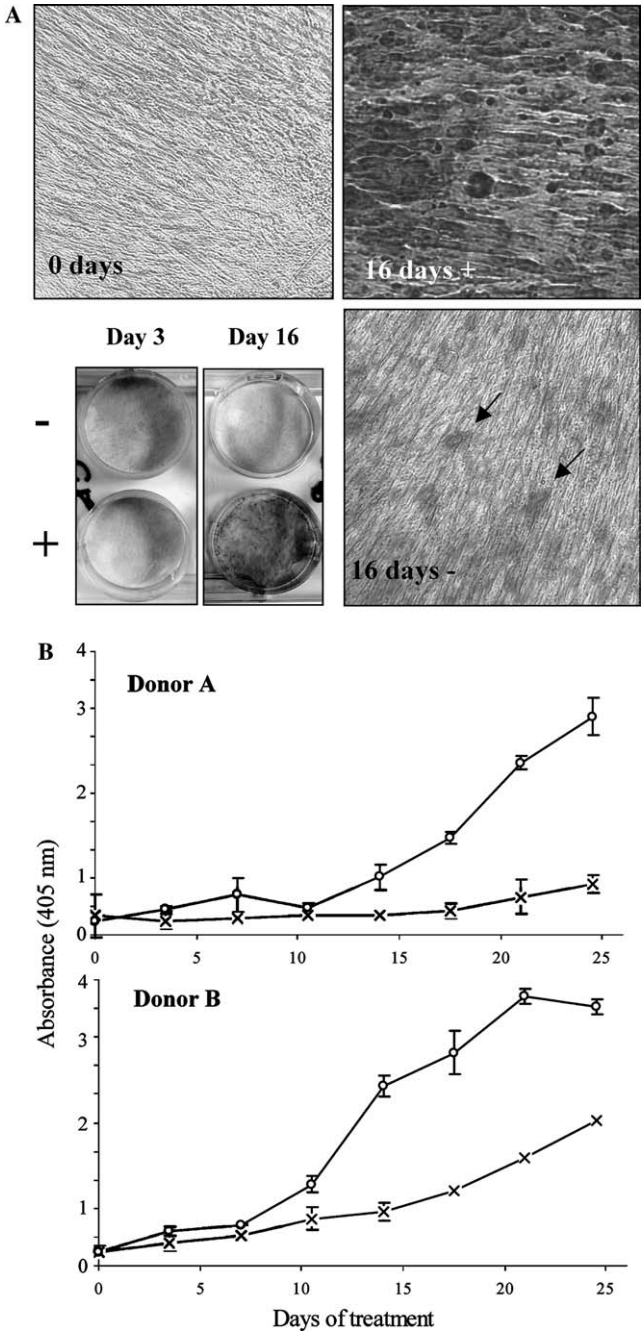

Fig. 4. (A) Phase microscopy of ARS-stained hMSCs mineralizing in culture. At day 0, there were no detectable mineral deposits on the confluent monolayer (0 day). In the presence of osteogenic supplements, small, densely stained nodules appeared from and coalesced into a continuous matrix by day 16 (16 days +). hMSCs spontaneously mineralized in the absence of osteogenic supplements to a much lesser degree (16 days −). After 16 days in the presence of osteogenic supplements, the monolayer was densely stained and visible by the naked eye (16 days +). (B) ARS acid extraction to semiquantify the production of mineral by hMSCs photographed in A. Open circles represent treatment of the cells with osteogenic medium. Crosses represent treatment of the cells with standard medium. Top and bottom plots represent data from two separate donors. Data are expressed as the mean ($n = 6$) with error bars representing ±1 SD.

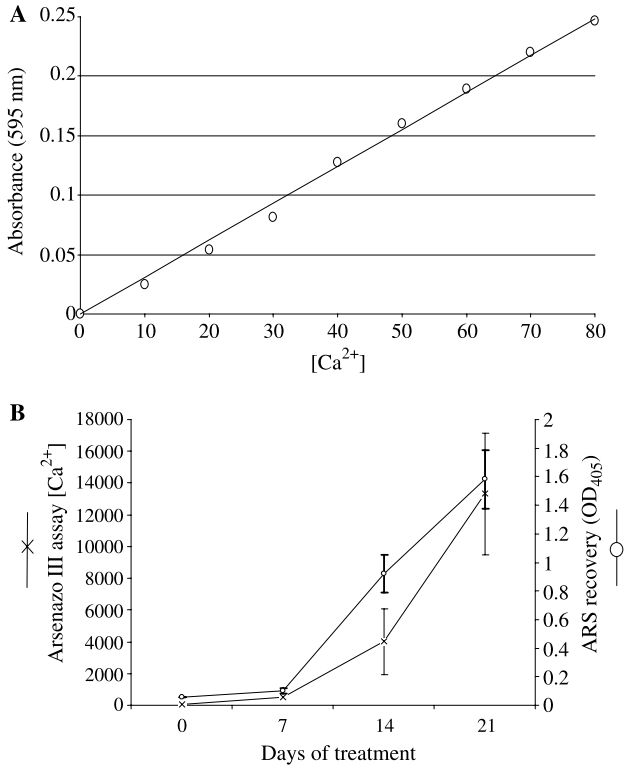

Fig. 5. Comparison of ARS acid extraction versus Arsenazo III assay after acid extraction on an hMSC osteogenic differentiation time course. (A) Standard curve demonstrating that the assay has a linear range up to 80 μM Ca^{2+}. (B) Time course of hMSC osteogenic differentiation assayed by ARS acid extraction or Arsenazo III assay after acid extraction. Data are expressed as the mean ($n = 6$) with error bars representing ±1 SD. The bolded error bars refer to the ARS-based assay and the fine error bars refer to the Arsenazo III assay.

HT1080 exhibited no detectable mineralization, but MG63, a well-documented osteogenic sarcoma cell line, rapidly accumulated mineral over the time in culture (Fig. 6). Interestingly, the LS1 cells also produced mineralized deposits, demonstrating that this osteosarcoma also consisted of osteogenic cells.

Discussion

This paper describes an accurate method for the assay of mineralization in monolayer cultures. It is based on Alizarin red S staining of the mineral followed by extraction with 10% acetic acid. The acidified ARS is then neutralized by the addition of ammonium hydroxide to reintroduce the red color. The color is then quantified in 96-well format by measurement of the absorbance at 405 nm. This assay is designed to provide a more sensitive alternative to CPC extraction for objective quantification of ARS-stained cultures. The data provide confirmation that phase contrast images are an accurate representation of the overall mineralized monolayer.

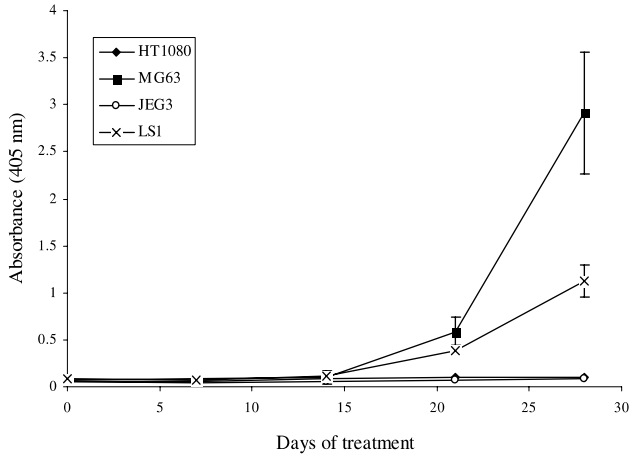

Fig. 6. Use of ARS extraction to semiquantify the production of mineral by MG63 osteogenic sarcoma cell line, JEG epithelial choriocarcinoma cell line, HT1080 hepatocarcinoma cell line and LS1, a cell line established from an osteosarcoma biopsy taken from a 12-year-old female. Data are expressed as the mean ($n = 6$) with error bars representing ±1 SD.

When compared with the alternative method for ARS extraction and quantification, CPC extraction at pH 7.0 and a traditional calcium detection assay based on colorimetric detection using Arsenazo III, the acid extraction procedure was found to be advantageous for numerous reasons. The acid extraction assay was found to be more reproducible with regard to experimental error than CPC extraction (Fig. 3) and more sensitive in that cetylpyridinium chloride failed to detect minor increases in mineralization, whereas the acid extraction method reproducibly detected the very subtle increases in mineral deposition in early osteogenic cultures (Fig. 3). When comparing the acid extraction assay with a traditional calcium detection protocol employing Arsenazo III, we found that the low and narrow linear range for the assay required extensive dilution of the samples that contributed to experimental variation (Fig. 5) and a more time-consuming procedure. Assays such as those that employ Arsenazo III [14,15] or cresolphthalein [16] are therefore more suited to assays of biological fluids containing modest levels of calcium. Prestaining of the cultures prior to extraction also provided major advantages over colorimetric measurement of extracts using established calcium assays in that the stained cultures may be visualized in detail prior to extraction so that micrographs and quantitative measurements can be directly related and the samples may be readily monitored throughout to assess color extraction.

Using the acid extraction assay, the kinetics of osteogenesis by hMSCs could be investigated, revealing that the cells deposited mineral very slowly over 7 days followed by a rapid phase of mineralization lasting 7–10 days. Thereafter, the deposition of mineral ceased (Figs. 3 and 4). This pattern is in agreement with the currently accepted mechanism of hMSC differentiation that predicts a lag phase where the cells progress through a number of intermediate stages prior to becoming a committed osteoblast [1,17]. The assay will be useful for assessment of the osteogenic properties of hMSCs from different donor sources and different aspirates from the same donor. These are both sources of variation at present and require further characterization [18]. The assay was also used to characterize the osteogenic properties of a number of established cell lines. Not surprisingly, cell lines derived from nonosteogenic tissue did not produce a detectable signal, whereas a well-characterized osteogenic cell line produced a substantially positive measurement when assayed. A cell line established from an osteoblastic tumor was tested for osteogenic properties ex vivo, confirming that the tumor was indeed mineralizing. Therefore, this method has utility in diagnosing mineralizing tumors derived from clinical biopsies. The report here describes a cost-effective and rapid method for the assay of mineral deposition in monlayer culture. It is more accurate and sensitive than the current colorimetric method used for such measurements and should be a valuable method for the quantification of osteogenesis.

Acknowledgments

This work was supported by grants from the American Cell Therapy Research Foundation, the National Institutes of Health (NIH AR47796 and AR 48323), the Oberkotter Foundation, HCA the Health Care Company and the Louisiana Gene Therapy Research Consortium.

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