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Computer-based image analysis of cartilage differentiation in embryonic limb bud micromass cultures

by JAMES C. GARRISON, PAUL PETERSON *and* EDWIN M. UYEKI,
Department of Pharmacology, Toxicology and Therapeutics, The University of Kansas Medical Centre, Kansas City, KS 66103, *U.S.A.*

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SUMMARY

A computer imaging system was used to analyse the effects of benzamide (BAM), sodium butyrate and dimethyl sulphoxide (DMSO) on chondrogenesis in culture. Embryonic chick limb bud cells were used as a source of undifferentiated mesenchyme, and cultures were prepared using a micromass culture technique. The degree of chondrogenesis is directly related to the amount of Alcian blue staining. Standard assays include either manual tally of individual cartilage nodules, which is both tedious and time-consuming, or extracting bound dye for spectrophotometric analysis, which obscures individual variations because it requires pooled samples. In the present study, low-magnification images of individual micromass colonies were converted into derived images based on their percentage transmittance relative to the background. These derived images were analysed for relative degree of chondrogenesis, using area and integrated optical density (IOD) as descriptors. While both types of data were useful for ranking the degree of chondrogenesis in culture, IOD was the preferred descriptor because of its accuracy over a wide range of threshold values. The area and IOD data both demonstrate that BAM produces marked enhancement of chondrogenesis in culture, sodium butyrate causes marked inhibition and DMSO produces mixed results (enhancement at the high dose, inhibition at the low dose). While the present study demonstrates the usefulness of computer-aided microscopy for analysis of low-magnification images, the same descriptors (area and IOD) should be useful in quantifying data from a variety of objects (cells, nuclei, etc.) which can be stained in a selected, quantitative fashion.

INTRODUCTION

The embryonic chick limb bud micromass technique (Ahrens *et al.*, 1977) is a valuable method for studying cartilage differentiation *in vitro*. Typically, spot colonies (3–5 mm in diameter) of undifferentiated chick limb bud cells are plated into culture dishes, exposed to agents which affect cell differentiation and assayed for cartilage on day 4. Standard assays for chondrogenesis are based on Alcian blue staining, and include either manually counting the number of cartilage nodules produced or extracting stain for spectrophotometric analysis. Both of these techniques have inherent weaknesses. Nodule counting provides the number of cartilage foci, but does not

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quantify the amount of cartilage synthesized. Spectrophotometry quantifies cartilage synthesis, but requires pooled samples for accuracy, thus obscuring individual variations among colonies (Garrison & Uyeki, 1988). In the present study we assayed the degree of differentiation of micromass cultures, treated with potential differentiating agents, using a novel computer imaging system that allows precise measurement of cartilage-bound Alcian blue dye based on the area and integrated optical density (IOD) of the colonies.

The present experiment was based on the observation that embryonic cells are similar to cancer cells in that both cell types are relatively undifferentiated and proliferate rapidly. Many of these undifferentiated cell types can be induced to display some degree of differentiated function in culture. Exposure to dimethyl sulphoxide (DMSO) (Friend *et al.*, 1971), sodium butyrate (Leder & Leder, 1975; Takahashi *et al.*, 1975) or nicotinamide (Morioka *et al.*, 1979) will induce Friend erythroid leukaemia cells to differentiate into haemoglobin-producing cells. Similarly, nicotinamide and a number of its analogues, including benzamide (BAM), have the ability to induce embryonic chick limb bud cells to differentiate into cartilage cells *in vitro* (Nishio *et al.*, 1983; Nakanishi & Uyeki, 1985). In both cases, the presumed mode of action is the same, namely, inhibition of poly(ADP-ribos)ylation (Morioka *et al.*, 1979; Nishio *et al.*, 1983). There have been no studies to determine whether DMSO or sodium butyrate can induce limb bud cell differentiation. Since the mechanism(s) of chemically induced cell differentiation has not been fully elucidated, but is thought to be the same for embryonic and transformed cells, it was important to know whether agents that induce differentiation among Friend leukaemia cells have a similar inducting effect on undifferentiated chick limb bud cells. We tested the effects of BAM, DMSO and sodium butyrate on chick limb bud cell differentiation *in vitro* in an attempt to answer this question. The use of computer imaging of the micromasses allowed rapid and precise data acquisition.

MATERIALS AND METHODS

Chemicals

Benzamide and sodium butyrate were purchased from Sigma Chemical Co., St Louis, MO. Dimethyl sulphoxide was purchased from Fisher Scientific, St Louis, MO. Alcian blue 8GX was purchased from Aldrich Chemical Co., Milwaukee, WI.

Cell culture

Limb bud cells were obtained from specific pathogen-free (SPF) Cofal negative White Leghorn chicken eggs (Larson Lab-Vac Eggs, Inc., Gowrie, IA). Eggs were incubated at 39°C in a rotating-drum incubator (model 100, Humidaire Incubator Co., New Madison, OH). Wings and legs were removed from approximately forty-eight stage 23–24 chick embryos (Hamburger & Hamilton, 1951) and the cells dissociated enzymatically (0·125% trypsin, 0·1% collagenase and 0·005% DNase I in Hank's balanced salt solution) for 30 min at 37°C. To facilitate digestion, cells were mixed on a Vortex mixer at 5-min intervals throughout the incubation period. Cells were further dissociated mechanically by trituration, washed twice in growth medium (Ham's F12 supplemented with 5% foetal calf serum (K.C. Biological, Inc., Lenexa, KS) and penicillin/streptomycin/amphotericin B), filtered through two 20-μm mesh Nitex filters and resuspended at a final plating density of 2×10^7 cells/ml. Cells were grown in micromass cultures (Ahrens *et al.*, 1977; Nakanishi & Uyeki, 1985), 20 μl of cell suspension per micromass, in 12-well tissue cluster plates and maintained at 37°C in a water-jacketed incubator containing an atmosphere of 5% CO_2–95% air. Medium was replaced at 48 h, and the cultures terminated for assay after 96 h total incubation.

Assay

Cartilage production was assayed as a function of Alcian blue staining. Colonies were fixed for 30 min in 95% ethanol, followed by 30 min in 10% formalin and stained with Alcian blue 8GX (1%, pH 1·0) for 1 h. Colonies were thoroughly rinsed in distilled water followed by 3% acetic acid. Cartilage production was quantified by measuring the area and optical density of the Alcian-blue-stained region with a computer imaging system as described below.

Computer aided microscopy (CAM)

Computer-enhanced images were obtained and analysed using an interactive video imaging system developed by Cancer Imaging of the British Columbia Cancer Research Centre in Vancouver, BC. This component system (integrated software/hardware) utilizes a Nikon Optiphot microscope attached to a high-resolution CCD scanner (Datacopy camera, model 610). The camera is directly connected to a digital signal processor (Matrox model MVP-AT) installed in an IBM-AT computer. Image capture and processing are controlled by software designed as an integral part of the imaging system. Typical micromass colonies ranged from 3 to 5 mm in diameter. A low-magnification microscopic image (objective magnification = 1, NA 0·03 and projection lens magnification = 2·5) was captured and increased to a total display magnification of 25 (screen magnification factor 10), processed and displayed on an analog RGB monitor (Sony model PVM-1271Q). Pixel resolution of the capture image was 512 × 512 pixels, with an individual pixel resolution of approximately 8 μm. A grey scale was displayed in a range from 0 (black) to 255 (white).

All images were captured using a highly stable light source/power supply (Kikusui model PAD 16-18L) to ensure a uniform background light intensity level. Captured images were 'normalized' relative to a stored image of the background field. Normalization consists of reassigning brightness values for each pixel from their absolute values (0–255) to derived values ranging from 0 to 100 (equivalent to percentage transmittance). This procedure is described by the formula $T = (R/B) \times 100$, where T is the normalized (derived) brightness value, R the brightness value from the raw image and B the brightness value from the stored background image. Normalization served two purposes: first, correcting for slight irregularities in background illumination and second, allowing comparison of images captured under different illumination conditions. It should be noted that a new background image is required whenever light intensity, condenser position or magnification changes.

Individual micromass colonies were analysed for area and IOD of the stained region of the colony. The IOD is defined as the summation of optical density (OD) values for each pixel in the image (Joyce-Loebl, 1985). IOD values are a function of both stain intensity and colony area (i.e. the amount of Alcian blue dye bound to the colony). The stained portions of the micromass colonies were demarcated for analysis from unstained regions by determining the threshold brightness (grey scale) level between these two regions, and the IOD determined for the demarcated colony. The threshold can be determined using a gradient threshold histogram to separate the stained area from the background (Fig. 1) (Castleman, 1979). The threshold grey scale values examined in the normalized images in the present study ranged from 20 to 45 (of 100 total). A grey scale value of 35 most accurately defined the colony margin in the present study, as confirmed by threshold contouring. Since the derived image (T) represents the percentage transmittance, OD for each pixel is obtained directly from $OD = -\log T$, and IOD of the thresholded image from the summed OD values, i.e. $IOD = \Sigma OD$.

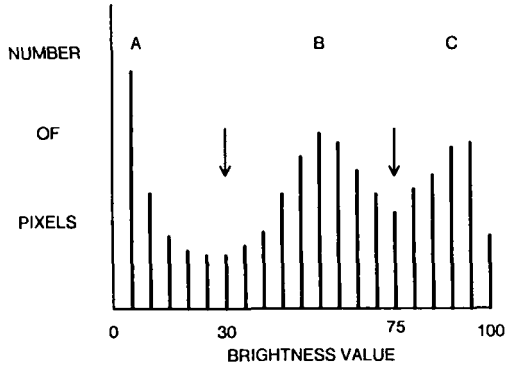

Fig. 1. Histogram of a typical Alcian-blue-stained micromass colony treated with a 0·5% DMSO, showing distribution of pixels at brightness values ranging from 0 to 100. Three distribution peaks are present, representing the stained region of the colony (A), the unstained portion of the colony, consisting primarily of undifferentiated cells (B) and the region of the culture plate devoid of cells (C). The threshold value between the stained and unstained regions is typically defined by the trough at brightness value 30. Similarly, a threshold between the unstained portion of the colony and the cell-free region could be defined by the trough at brightness value 75.

Statistics

Statistical significance was determined at the 0·01 and 0·05% level using Duncan's multiple comparisons test (Bruning & Kintz, 1987).

RESULTS

Prior to image analysis, several basic questions were addressed. (1) Is colony area an adequate descriptor of chondrocytic differentiation? (2) If so, how sensitive is area (versus IOD) as a means of measuring chondrogenesis in the micromass assay? (3) How does improper thresholding influence the data? To answer these questions, we measured the area and IOD of each image (five colonies per treatment, twelve treatment groups, for sixty images total), using six threshold values for each measurement (twelve total measurements per image). Measurements were taken at grey scale threshold values of 45, 40, 35, 30, 25 and 20. A high threshold value (45) caused inclusion of the unstained area, while a low threshold value (20) excluded portions of the stained area (Fig. 2).

Area and IOD data showed similar patterns (Fig. 3). The range of values (percentage variation within treatment groups) was greater for area than IOD. For this reason, it was of critical importance to determine the proper threshold for valid area comparisons. While a gradient threshold histogram will usually define the proper threshold value, direct observations of the micromass image (threshold contour) are required to ensure that an accurate colony boundary has been demarcated. When properly thresholded, area usually was an adequate descriptor to determine significant differences between groups (Fig. 4). Indeed, in the case of the colonies treated with 0·3 mM BAM, area appeared to be a better descriptor than IOD because of the uniformity of colony size. However, the area data were not as 'tight' for the colonies treated with DMSO or butyrate (compare area and IOD measurements among 0·03 mM butyrate-treated colonies, Fig. 3).

Improper thresholding revealed the inherent weakness of using area measurements to describe the degree of chondrogenesis *in vitro* (Fig. 3). A worst case example of the

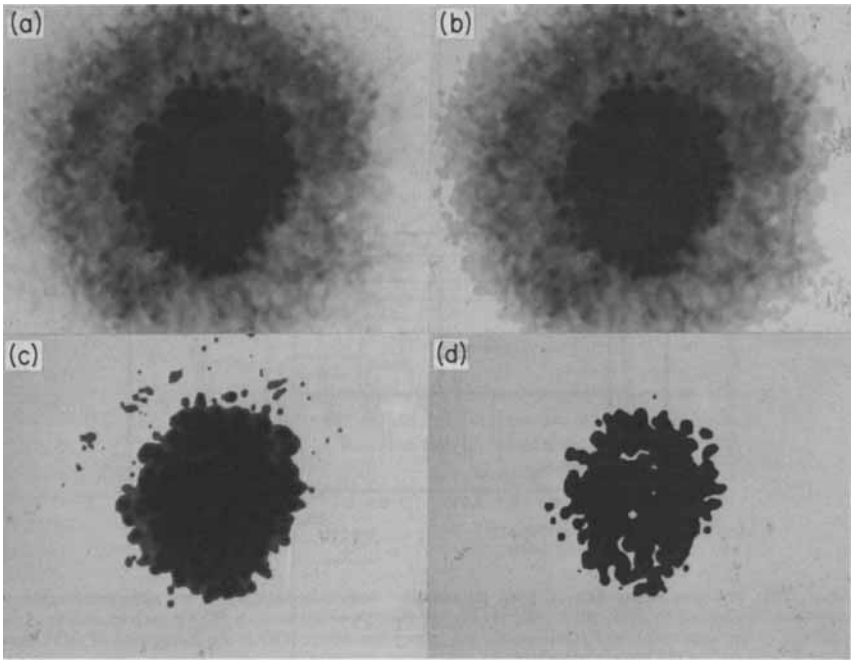

Fig. 2. A typical micromass colony threshold using different brightness values to define the colony boundary for area and IOD determinations. (a) Normalized image prior to thresholding. (b) Image produced using an excessively high threshold grey-scale value (45), showing inclusion of background area. (c) Image produced using a threshold grey-scale value of 35, accurately demarcating the colony boundary. (d) Image produced using unacceptably low threshold grey-scale value (20), showing loss of colony periphery.

difference between area and IOD measurements can be seen when comparing colonies treated with 0·3 and 1·0 mM BAM. At a threshold of 45, the IOD of colonies treated with 0·3 mM BAM is significantly lower (0·01 level) than those treated with 1·0 mM BAM. Conversely, the area of colonies treated with 0·3 mM BAM is significantly higher (0·05 level) than those treated with 1·0 mM BAM. Direct observations of the areas bounded at this threshold level indicate these are not valid area measurements because of the inclusion of unstained background in the measured area.

IOD measurements gave consistent results, regardless of the threshold value chosen (Fig. 3). Inclusion of the unstained area surrounding the colony had a minor impact on the final values, since this portion was of very low density. Measurement of IOD demonstrated a dose–response effect to all three test compounds (Fig. 4). Chondrogenesis was enhanced by BAM (1·0 and 3·0 mM) and DMSO (1·5%), while butyrate caused inhibition at all doses. The enhancement by BAM was dramatic, particularly among cultures treated at the highest dose where the IOD value was four times that of the controls (165,108 versus 41,831). Interestingly, the lowest dose of DMSO (0·05%) inhibited chondrogenesis.

DISCUSSION

This study demonstrates the usefulness of computer imaging for analysing the degree of chondrocytic differentiation in cell culture. The use of a procedure which

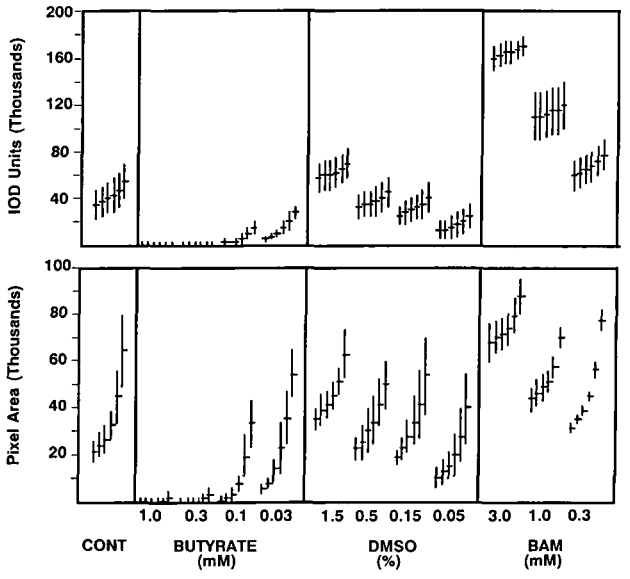

Fig. 3. IOD and area values derived from micromass colonies thresholded at six different brightness (grey-scale) levels (left to right, 20, 25, 30, 35, 40, 45). Bars show the mean ± SD for each treatment. Area is defined as the total number of pixels within the colony boundary. IOD is the summation of OD values for each pixel within the colony boundary.

converts raw-image brightness values into transmittance-value equivalents allows the acquisition of uniform images, despite some irregularities in background illumination. Potentially, this procedure is also useful for 'decoupling' the acquired OD values from the absolute brightness of the illumination source; i.e. regardless of the intensity of the background illumination the derived images can be compared one to another. There are, of course, limits to the usefulness of 'normalization'. Raw images captured with low-intensity illumination may lack detail compared to the same images captured at higher intensities. Since these low-intensity images are displayed over a narrow range of brightness (grey scale) values, areas of similar (but not necessarily identical) density are combined. In addition, changes in illumination which influence contrast may also influence IOD. To ensure that images are directly comparable, controlled illumination conditions should be maintained throughout the period of image capture. In general, our procedure is most effective when contrast levels are adequate to show a marked separation of the micromass colony from the unstained background.

Using both area and IOD determinations, data was acquired which indicated the relative degree of cartilage differentiation *in vitro*. In the present study, area was as effective as IOD for demonstrating the degree of chondrogenesis, as long as threshold grey-scale values accurately demarcated the margins of the colonies. It should be noted, however, that the relative range in values was greater for area than IOD (Fig. 3). The usefulness of area as a descriptor of chondrogenesis is a function of colony age and cartilage distribution patterns. That is, as early chondrogenesis proceeds *in vitro*, successively greater areas of the colony become stained. Thus, during early colony formation there is a direct relationship between area and the degree of chondrogenesis. However, area is generally a poor descriptor of later chondrogenesis. Once

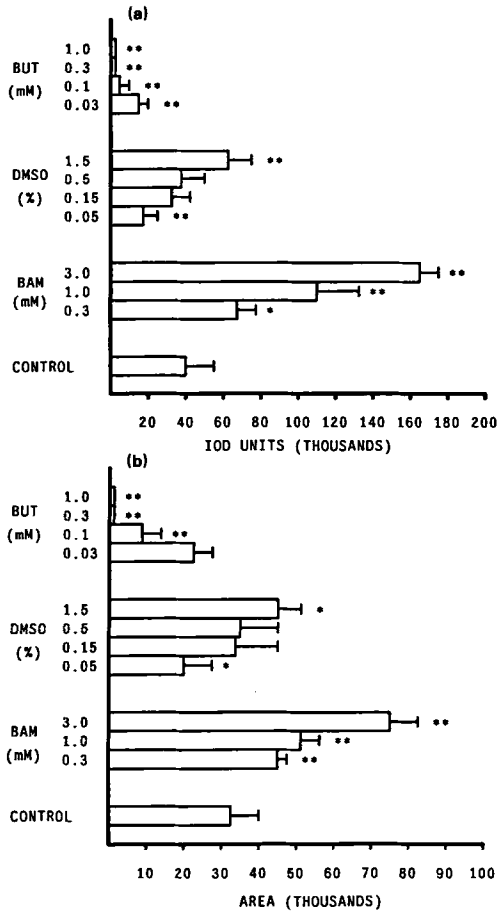

Fig. 4. IOD (a) and area (b) values derived from micromass colonies thresholded at a brightness (grey-scale) level of 35. Bars demonstrate mean ± SD for each treatment. Values significantly different than controls are marked (*P = 0·05, **P = 0·01).

cartilage nodules fuse together (day 4–5), further increases in colony area are minimal, even though cartilage deposition continues.

IOD is a quantitative descriptor of the degree of staining within a defined area, incorporating both density and area information into a single value. When measuring IOD, high-density areas of chondrocyte cultures are heavily weighted in comparison to low-density areas. This is shown in Fig. 3, where increasing the threshold value often causes large increases in area but only moderate increases in IOD. This aspect of IOD is important as it allows colonies to be assayed over a relatively wide range of threshold values and also allows assay of continued chondrogenesis after colony size has stabilized. In essence, while area is a 2-D descriptor, IOD incorporates information from a third dimension. Previous work demonstrated that the IOD method is comparable or superior to methods typically used to assay cartilage differentiation

in vitro (Garrison & Uyeki, 1988). Standard micromass assays include counting the number of cartilage nodules or extracting bound Alcian blue dye for spectrophotometric analysis. These standard procedures have inherent weaknesses. Nodules frequently fuse together, making counting both difficult and inaccurate. Spectrophotometry, analogous to IOD, requires the pooling of a number of colonies to extract enough dye for measurement, hence, obscuring individual variation among colonies.

A major purpose of this study was to use digital image processing to evaluate embryonic cell differentiation *in vitro*, and compare the effects of chemical agents known to induce differentiation in a passaged cell line (Friend leukaemia). Among the agents useful in promoting cell differentiation of Friend leukaemia cells are DMSO, BAM and sodium butyrate. Thus, it was of interest to determine how these agents affected the course of cell differentiation in dispersed limb bud cell culture. BAM caused a marked increase in cartilage differentiation, as previously reported (Nishio *et al.*, 1983, Nakanishi & Uyeki, 1985). DMSO induced cartilage differentiation at higher doses, while at low doses it inhibited cartilage differentiation. Butyrate caused a marked inhibition of chondrogenesis. It was particularly interesting that 1·5% DMSO caused a marked increase in cartilage production, since this is a toxic dose to many cell types, including Chinese hamster ovary cells (E. Uyeki, unpublished data). Friend *et al.* (1971) demonstrated that 2% DMSO produced maximal stimulation in Friend leukaemia cells, but also produced some toxicity during the first few days of culture. Our previous experience with chick limb bud cells has shown that concentrations of DMSO in the range of 3–4% are toxic. The relationship between DMSO toxicity and cell differentiation is not understood at present. The inhibitory effect of sodium butyrate on chondrocytic differentiation is in direct contrast to its stimulatory effect in Friend leukaemia cells (Leder & Leder, 1975; Takahashi *et al.*, 1975), suggesting that there are significant differences in the way these two cell types respond to presumptive differentiating agents.

The results from the present study clearly indicate that BAM, DMSO and sodium butyrate produce a different range of effects in our primary chick cell culture than in Friend leukaemia. We feel that a further comparison of these three agents on the limb bud culture may provide us with additional clues regarding the triggers/cues which turn on/off cell differentiation.

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