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DMSO regulates osteoclast development in vitro

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Abstract

Dimethyl sulfoxide (DMSO) is routinely used in the laboratory as a solvent and vehicle for organic molecules. Although it has been used in previous studies involving myeloid cells and macrophages, we are unaware of data demonstrating the effects of DMSO alone on osteoclast development. Recently, we were using DMSO as a vehicle and included a non-vehicle control. Surprisingly, we observed a marked change in osteoclast development, and therefore designed this study to examine the effects of DMSO on osteoclast development. Osteoclasts were generated from two sources: bone marrow macrophages and an osteoclast progenitor cell line. Cells were cultured with DMSO for various durations and at differing concentrations and mature, multinucleated (>3 nuclei) TRAP⁺ cells were assessed in terms of cell number, cell surface area, and number of nuclei/cell. Osteoclast surface area increased in 5 μ M DMSO to a mean of 156,422 pixels from a mean of 38,510 pixels in control culture, and subsequently decreased in 10 μ M DMSO to a mean of 18,994 pixels. With serial addition of DMSO over 5 d, a significant increase in mean surface area, and number of nuclei/cell was also observed, while the opposite was true when DMSO was serially removed from culture. These findings show that DMSO exerts a marked effect on osteoclast differentiation. Since many investigators use DMSO to solubilize compounds for treatment of osteoclasts, caution is warranted as altering DMSO concentrations may have a profound effect on the final data, especially if osteoclast differentiation is being assessed.

Keywords

Dimethyl sulfoxide; Osteoclast; Differentiation; Maturation

Introduction

Dimethyl sulfoxide (DMSO) is an amphipathic organic compound with a high dielectric constant (Rammler and Zaffaroni 1967) bearing chemical properties that afford it many biologic applications. This compound has been demonstrated to alter membrane fluidity (Lyman et al. 1976; Friend and Freedman 1978) and permeability (Fowler et al. 1961; Jacob et al. 1964), making it especially effective as a carrier for pharmaceuticals, electrolytes, and other molecules (Stoughton and Fritsch 1964; Kligman 1965a, b; Franz and Van Bruggen 1967; MacGregor 1967; Narula 1967; Keil 1975). It has also been shown to modify enzyme activity (Sams and Carroll 1966; Rammler 1967), change the secondary structure of both DNA and RNA, affect the mitotic cycle of normal dividing cells (Hervas and Gimenez-Martin 1973), and impede cell membrane-bound electron transfer systems (Archer et al. 1967).

This versatile compound has been shown to induce differentiation of myeloid cell lines into mature granulocytes or macrophage-like cells (Preisler and Lyman 1975; Collins et al. 1978; Collins et al. 1980; Myers and Siegel 1984; Hassan 1988; Nakamura et al. 1990; Kitajima et al. 2001). DMSO has also been demonstrated to potentiate apoptotic signaling pathways involving TNF- α and NF- κ B (Depraetere et al. 1995; Ohashi et al. 2000; Vondracek et al. 2001; Vondracek et al. 2006), perhaps due to the effects of the compound on the mitochondrial membrane (Santos et al. 2003; Vondracek et al. 2006) or with respect to the Apo2L/TRAIL protein (Kelly et al. 1994; Ashkenazi and Dixit 1998). In addition, DMSO has been shown to have an overall inhibitory effect on cell proliferation (Brennan et al. 1991; Takase et al. 1992; Marthyn et al. 1998; Egli et al. 2003).

Due to its utility as a solvent, many studies involving the cell biology of bone have incorporated DMSO into their experimental protocols (Komarova et al. 2003; Rawadi et al. 2003; Aoki et al. 2006; Saito et al. 2007), as DMSO has heretofore been believed to be an innocuous reagent. However, in collecting data for a study on the effects of 18 α -glycyrrhetic acid for inhibition of gap junction intercellular communication, we discovered that the addition of DMSO to culture preparations exerted a direct and marked effect on the characteristics of the osteoclasts in culture, and therefore could drastically alter the results of our experiment. Despite the documented effects of this compound on myeloid-derived cells, to our knowledge, no research has been published in the literature to examine its effects on osteoclasts. Intrigued by our surprising finding, we endeavor in this study to clarify the influence DMSO bears on osteoclast development, at varying concentrations and for various durations.

Materials and Methods

In vitro osteoclast-like cell formation models

Osteoclast-like cells were generated by previously described methods (Udagawa et al. 1989; Yasuda et al. 1998; Kacena et al. 2006). In brief, 100,000 osteoclast progenitors from the Pax5 $^{-/-}$ spleen cell line (a spleen cell line derived from Pax5 $^{-/-}$ mice which has been previously described (Horowitz et al. 2004) or primary c57BL/6 mouse bone marrow macrophages were cultured in α -MEM supplemented with 10% FCS (Hyclone, Logan, UT or Atlanta Biologicals, Lawrenceville, GA) and 30 ng/ml of M-CSF (R&D Systems, Minneapolis, MN) and 50 ng/ml RANKL (R&D Systems). Media was changed every third day for 4–8 d (until osteoclasts were formed). In each of the above models, once osteoclasts had formed, the cells were fixed with 2.5% glutaraldehyde in phosphate buffered saline for 30 min at room temperature, stained for TRAP using a kit from Sigma (St. Louis, MO) and TRAP $^{+}$, multinucleated (>3) osteoclast cells were counted. DMSO (Fisher Scientific, Pittsburgh, PA or J.T. Baker, Phillipsburg, NJ) was titrated into cultures (0–40 μ M).

Morphometric measurements

After counting the total number of all the TRAP⁺, multinucleated (>3 nuclei) osteoclast cells in each well, four representative pictures, one from each quadrant, were taken per well under $\times 10$ magnification. ImageJ (National Institute of Health) was used to count the number of nuclei and measure the surface area of each TRAP⁺, multinucleated (>3 nuclei) osteoclast cells. The cell counter plug-in was used to count and keep track of the number of nuclei per cell; freehand selections tool was used to trace the outline of the cell, and the measure function under the analyze tab was used to measure the surface area (in pixels) of each selected cell.

Statistical analysis

Unless otherwise stated, all data are presented as the mean $\pm$ 1 SD. For studies when DMSO concentration alone was analyzed, one-way ANOVA was used to determine significant differences ($p < 0.05$). Two-way factorial analyses of variances were used to compare groups, with duration of exposure and DMSO concentration being the independent variables. In the event of a significant interaction, pair-wise Bonferroni comparisons were made to explore individual group differences while controlling for the elevated family-wise error associated with performing multiple comparisons. All analyses were performed with the Statistical Package for Social Sciences (SPSS 6.1.1; Norusis/SPSS Inc., Chicago, IL) software and were two-tailed with a level of significance set at 0.05. Within individual experiments, data points are based on a minimum of triplicate representative samples and experiments were repeated at least once.

Results

Variation in osteoclast characteristics with varying concentrations of DMSO

Osteoclast number significantly increased in cultures containing 5 μ M DMSO as compared with control cultures without DMSO. However, osteoclast number declined with higher concentrations of DMSO (10 μ M), and osteoclasts were completely absent in cultures containing 20 or 40 μ M DMSO (Figs. 1 and 2). The average nuclei and surface area/cell (1,345 pixels=1 mm) also followed a similar pattern (Fig. 2). It should be noted that in preliminary studies not shown here, we also examined 1 and 2.5 μ M DMSO and found that 1 μ M DMSO did not have an apparent effect compared with untreated controls, but 2.5 μ M DMSO samples had significantly more osteoclasts than did untreated controls, but significantly fewer than that observed with 5 μ M DMSO.

Of importance, with the exception of the highest dose tested, viable cell number was not altered by DMSO. As an example, total viable cell number (multinucleated osteoclasts and all other cells) was 329 $\pm$ 98 with 0 μ M DMSO, and 371 $\pm$ 106 with 20 μ M DMSO. However, the total viable cell number dropped significantly in cultures containing 40 μ M DMSO (143 $\pm$ 30; $p=0.0004$).

As shown in Fig. 1B, osteoclast surface area was significantly increased with just 5 μ M of DMSO. Table 1 summarizes these findings. Cell surface area (in pixels) for control cultures containing no DMSO ranged from 4,180 to 133,207 with a median and average of 25,126 and 38,510, respectively. With 5 μ M of DMSO, cell surface area was significantly increased compared with controls with a range of 6,306–888,901, and a median and average of 96,028 and 156,422, respectively. With 10 μ M of DMSO, cell surface area decreased below control levels with a range of 2,094–78,681 and a median and average of 13,036 and 18,994, respectively.

Interestingly, as depicted in Table 1, 71% of control osteoclasts (0 μ M DMSO) and 89% of osteoclasts cultured with 10 μ M DMSO had a surface area which was $\leq 50,000$ pixels, whereas only 30% of osteoclasts cultured with 5 μ M DMSO were $\leq 50,000$ pixels in size. On the other hand, 48% of the osteoclasts treated with 5 μ M DMSO had a surface area which was $>100,000$ pixels as compared with 9% and 0% seen in control and 10 μ M DMSO cultures, respectively. The largest cells measured at each concentration were 133,207 (0 μ M DMSO), 888,901 (5 μ M DMSO), and 78,681 (10 μ M DMSO). This represents a 6.7 to 11.3 fold increase in surface area in the 5 μ M DMSO concentration compared with control and 10 μ M DMSO cultures, respectively.

Osteoclast numbers in response to adding and removing DMSO

As detailed in Fig. 3, in each experiment there are two controls: (1) cultures containing no DMSO throughout the experimental period; and (2) cultures containing 5 μ M DMSO throughout the experimental period. In other cultures, 5 μ M DMSO was either added on day 1 through day 4 of the experiment (Fig. 3A–C), or added on day 0 and removed on day 1 through day 4 of the experiment (Fig. 3D–F). Osteoclast number (Fig. 3A, D), nuclei/cell (Fig. 3B, E) and surface area/cell (Fig. 3C, F) were determined as in the previous experiments. Regardless of when DMSO was added or removed from cultures (Fig. 3A, D), in all cases as long as cells were not exposed to DMSO for the entire culture duration, the number of osteoclasts formed was unaltered and was not significantly different from the number of osteoclasts observed in control cultures without DMSO. However, osteoclast number was significantly higher in cultures containing 5 μ M DMSO for the entire culture duration as compared with those containing DMSO for a shorter number of days (any duration) or compared with control cultures without DMSO.

Osteoclast characteristics in response to adding and removing DMSO

Cultures containing DMSO for the entire experimental duration had significantly more nuclei/cell (Fig. 3B, E) and a larger surface area/cell (Fig. 3C, F) as compared with controls without DMSO. In the experiment, where DMSO was added on days 1 through 4 of the experiment, the following trend was observed: both nuclei/cell (Fig. 3B) and surface area/cell (Fig. 3C) increased with culture duration. For nuclei/cell (Fig. 3B), the effect was more pronounced when DMSO was added during later stages of the experiment. Specifically, the increase in nuclei/cell became significantly higher when DMSO was added during day 2 or later compared with control cultures containing no DMSO, and significantly higher when DMSO is added during day 3 or later compared with control cultures containing DMSO. Of note, the differences between DMSO added to cultures daily compared with control cultures with DMSO in surface area/cell (Fig. 3C) mirrored that of nuclei/cell (Fig. 3B).

On the other hand, when DMSO was added on day 0, then subsequently removed on days 1 through 4, the following trend was observed: both nuclei/cell (Fig. 3E) and surface area/cell (Fig. 3F) initially increased, then decreased with culture duration. Specifically, there was initial increase in both nuclei number/cell (Fig. 3E) and surface area/cell (Fig. 3F) (significant compared with both control cultures containing DMSO for the entire experiment and control cultures not containing DMSO) followed by a drop which ended at a level below or equivalent to that measured in cultures containing DMSO for the entire duration. This result is not completely surprising as in the cultures where DMSO was removed on day 4, DMSO is removed only 1 d prior to cell fixation.

Discussion

DMSO bears remarkable properties as an organic solvent and cryoprotectant. While this compound has been utilized in study protocols throughout the scientific literature involving

osteoclasts, its effects on these cells is poorly understood. DMSO is used to dissolve a number of inhibitors used in studying osteoclasts such as: PP2 (src inhibitor), PI3K inhibitors (wortmannin and Ly294002), and c-fms tyrosine kinase inhibitor (Ki20227). In most cases the cell cultures contain 0.1–0.5% DMSO (Hayashi et al. 2003; David et al. 2005; Park et al. 2005; Ohno et al. 2006; Zhou et al. 2006; Chang et al. 2008; de Vries et al. 2009). Of note, 0.5% DMSO is equivalent to our 5 μ M DMSO group where osteoclast number, size, and nuclei were all significantly elevated. The 0.1% DMSO is equivalent to 1 μ M DMSO which in preliminary studies we found did not result in significant changes compared with untreated controls (data not shown). Thus, since the data presented here demonstrate that 5 μ M DMSO (0.5%) exerts a marked effect on osteoclast formation, differentiation, and morphology, it may be important to consider the effectiveness of the inhibitors in this “enhanced” culture condition and/or if feasible it may be advisable to reduce the DMSO volume to 0.1% of the total volume.

In cultures containing 5 μ M DMSO, a significant increase in osteoclast induction was observed, while in the presence of 10 μ M DMSO, fewer mature osteoclasts were seen than in untreated control cultures, and no TRAP⁺ osteoclasts were observed in cultures containing >20 μ M DMSO. These results may indicate that a concentration of DMSO at or near 5 μ M is optimal for induction of osteoclast differentiation, while higher concentrations (>20 μ M) may exert a net inhibitory effect on osteoclasts.

Interestingly, variation in the concentration of DMSO present in culture largely had no effect on viable cell number. Cultures containing 0–20 μ M DMSO showed no significant difference in the number of viable cells, while cultures with 40 μ M DMSO yielded considerably fewer viable cells, likely undergoing apoptosis as described by other investigators (Depraetere et al. 1995; Ohashi et al. 2000; Vondracek et al. 2001; Vondracek et al. 2006).

Similar effects of DMSO were observed with respect surface area of mature osteoclasts on morphometric analysis. Osteoclasts grown in the presence of 5 μ M DMSO demonstrated considerably larger surface areas in comparison to those grown in untreated control cultures. As with number of osteoclasts formed, cell surface area decreased dramatically with a higher concentration of DMSO. In general, 5 μ M DMSO produced substantially larger osteoclasts in terms of surface area when compared with those cultured with 0 and 10 μ M DMSO. This may similarly indicate that 5 μ M DMSO is a near optimal concentration for the differentiation and maturation of osteoclasts.

With regard to cultures both absent of and containing 5 μ M DMSO at the outset (Fig. 3), neither the addition nor removal of DMSO from these cultures demonstrated any effect on the number of osteoclasts formed as compared with control cultures not containing DMSO (Fig. 3A, D). Consistent with data contained in Fig. 2, when DMSO is present for the entire culture duration, significantly higher numbers of osteoclasts were observed as compared with control cultures without DMSO.

Analysis of cell morphology in these cultures also showed an overall increase in cell surface area with the addition of DMSO, with an acceleration of this phenomenon after 2 d of serial addition. Likewise, the number of nuclei per cell increased with addition of DMSO in a similar fashion. The removal of DMSO had the opposite effect with regard to surface area and number of nuclei. These data indicate that 5 μ M DMSO is a potent inducer of osteoclast differentiation.

It should be noted that in these studies, we used two brands of DMSO, two separate brands of serum, and we tested both primary cells and a cell line. In all cases, similar data trends were seen. For example, in all cases 5 μ M DMSO induced osteoclast differentiation

compared with untreated controls and in all cases further increasing DMSO concentration resulted in a decline in mature osteoclast formation. However, the degree of change varied. As an example, in studies using the Pax5^{-/-} spleen cell line a more dramatic reduction in the number of osteoclasts formed was observed between 5 and 10 μ M DMSO, whereas when primary bone marrow macrophages were utilized, although a significant reduction was observed between 5 and 10 μ M DMSO the largest reduction was observed between 10 and 20 μ M DMSO.

Conclusions

Here, we demonstrate that DMSO exerts a marked effect on osteoclast differentiation. As many studies in the literature involving osteoclasts have utilized DMSO as a vehicle for testing compounds of interest, it is important to consider that the compound's effect on osteoclasts may vary based on the concentration of DMSO used. Therefore, caution is warranted when utilizing DMSO as a solvent for reagents as the DMSO itself, depending on the concentration, may differentially impact osteoclast formation and differentiation.

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Figure 1.

Osteoclast development in response to increasing concentrations of DMSO. Cells were harvested, fixed, stained for TRAP activity, viewed under a microscope, and photographed at $\times 10$. Control cultures contained no DMSO (A). Significant increases in osteoclast size was observed when cells were cultured with 5 μM DMSO (B). Subsequently, significant decreases in osteoclast size, to that of control levels, was observed when cultures contained 10 μM DMSO (C). Finally, no osteoclasts were observed in cultures containing 20 μM DMSO (D).

Figure 2.

Osteoclast (OC) development in response to increasing concentrations of DMSO. Cells were harvested, fixed, stained for TRAP activity, viewed under a microscope, and photographed at $\times 10$ magnification. The number of TRAP⁺ osteoclasts with >3 nuclei (A), nuclei/cell (B), and surface area/cell (C) were all significantly elevated in cultures containing 5 μM DMSO. Further increases in DMSO concentrations resulted in a significant reduction or complete absence in osteoclast number and simultaneous reductions in nuclei/cell and surface area/cell. Scale for surface area is 1,345 pixels=1 mm.

Figure 3.

The effect of DMSO on osteoclast (OC) development with regard to duration and timing of exposure. Cells were harvested, fixed, stained for TRAP activity, viewed under a microscope, and photographed at $\times 10$ magnification. The effect of 5 μ M DMSO exposure on osteoclast number (A and D), nuclei/cell (B and E), and surface area/cell (C and F) were determined when 5 μ M DMSO was added (A–C) or removed (D–F) from the cultures on days 1–4. There are two controls for each study, one group did not contain DMSO throughout the experiment, and one group contained DMSO throughout the experiment. Scale for surface area is 1,345 pixels=1 mm.

Table 1

Concentration dependent changes in surface area of osteoclasts

Concentration	% of cells in size range					Range	Average	StDev	Median
	≤10,000	10,001–50,000	50,001–100,000	>100,000					
0 μM	31	40	20		9	4,180–133,207	38,510	38,764	25,126
5 μM	3	27	22		48	6,306–888,901	156,422	183,615	96,028
10 μM	38	51	11		0	2,094–78,681	18,994	17,403	13,036