

Role of Acetylcholinesterase in the Regulation of Mesenchymal Stem Cell

Proliferation and Differentiation

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INTRODUCTION

Mesenchymal stem cells (MSCs) play a key role in the regulation of tissue maintenance and repair. These cells have multipotent potential *in vivo*, which can be maintained when they are cultured *in vitro*. MSCs can be derived from various tissues including bone marrow, umbilical cord, Wharton's jelly of the umbilical cord, placenta, adipose tissue, dental pulp, as well as from the lungs of lung transplant patients. *In vitro*, these cells are adherent to tissue culture flasks, display a fibroblast-like morphology, and maintain the ability to differentiate into osteoblasts, adipocytes, and chondroblasts. It has been demonstrated that MSCs are also capable of differentiating into cell types of different germ layer origins including neurons, alveolar epithelium, hepatocytes, and myocytes. Currently, only a few signaling pathways involved in the regulation of MSC proliferation and differentiation have been identified (e.g. Wnt signaling pathway). Disruption of these pathways drastically alters the ability of MSCs to proliferate and differentiate, therefore, altering the ability of MSCs to participate in tissue maintenance and/or repair. Service members have the potential to be exposed to many different toxic chemicals. These chemicals could disrupt the normal functions of MSCs and interruption of the normal healing processes is of concern for injured service members preparing to return to duty. Recently, it has been reported that bone marrow-derived MSCs express active acetylcholinesterase (AChE) and that disruption of this activity by organophosphate chemicals affects the ability of MSCs to differentiate into osteoblasts. The role AChE plays in MSC proliferation and differentiation is currently unknown. In this study, we examined the effects of organophosphates on AChE activity, MSC proliferation, as well as MSC differentiation.

MATERIALS AND METHODS

Experimental Chemicals: Stock solutions of the organophosphate pesticides (OPP) parathion and paraoxon were prepared in 100% ethanol (EtOH). The cholinesterase reactivator pralidoxime (2-PAM) was prepared in sterile deionized water and stored at 4°C.

Human MSC Culture: Primary human bone marrow-derived MSCs were obtained from Lonza (Walkersville, MD) and cultured in Mesenchymal Stem Cell Growth Medium. Only MSCs from passages 4-8 were used.

Parathion/Paraoxon Toxicity Studies: We plated 1x10⁴ MSCs in the wells of 96-well tissue culture plates and allowed the cells to attach for 24h. MSCs were then exposed to increasing concentrations of parathion, paraoxon, or vehicle (EtOH) for 48h. A MTT Cell Viability Assay was then performed according to the manufacturer's protocol. Results were read on a SpectraMax[®] Plate Reader and expressed as % relative viability. For AChE reactivation studies, MSCs were exposed to media alone, vehicle (EtOH), 2-PAM, Paraoxon or Paraoxon+2-PAM for 24h. The cells were then evaluated as stated.

MSC Proliferation Studies: To evaluate the effects of parathion/paraoxon on MSC proliferation, we plated 5x10³ MSCs in the wells of 96-well tissue culture plates and allowed the cells to attach for 24h. Next, we exposed the MSCs to increasing concentrations of parathion, paraoxon, or vehicle (EtOH) for 48h. MSC proliferation was then evaluated using the BrdU Cell Proliferation Assay according to the manufacturer. Plates were then read on a SpectraMax[®] Plate Reader and results expressed as percent BrdU incorporation.

Determination of AChE Activity: AChE activity within the MSCs was measured using the colorimetric AChE Assay Kit according to the manufacturer's protocol.

Determination of AChE Expression in MSCs (Western blotting): Confluent MSCs were exposed to parathion, paraoxon, or vehicle (EtOH) for 24h. Then, the MSCs were lysed and collected in 1X RIPA buffer, centrifuged, and the supernatants assayed for protein concentration with the Pierce[™] 660nm Protein Assay. The samples were resolved using the 4-12% gradient Bolt[™] Bis-Tris Plus Gel according to the manufacturer's instructions and transferred onto nitrocellulose membranes using the iBlot[™] 7-Minute Blotting System. The membranes were blocked, incubated with rabbit anti-AChE antibodies, and incubated with anti-rabbit IgG antibodies conjugated to alkaline phosphatase using the iBlot[™] Western Blot System according to the manufacturer's instructions. Finally, the membranes were developed using the iBlot[™] Western Detection, Chromogenic Kit. To ensure equal loading, equivalent samples were run and blots probed with anti-GAPDH antibodies and processed as stated above.

Adipogenic Differentiation of MSCs: MSCs were plated in 96-well tissue culture plates at a density of 5x10³ cells/well and allowed to grow to confluence. The media was replaced with Human MSC Adipogenic Induction Medium and Adipogenic Maintenance Medium alone or with parathion, paraoxon, or vehicle (EtOH) in cycles in accordance with the manufacturer's protocol, and cultured for 7-21d. The cells were stained using the AdipoRed[™] Assay Reagent protocol and measured using a microplate fluorescence spectrophotometer. Results were expressed as relative fluorescence units (RFU). MSCs were also evaluated for adipogenic differentiation by staining with Oil Red O followed by image analysis using ImageJ software (NIH).

Osteogenic Differentiation of MSCs: MSCs were plated in 96-well tissue culture plates at a density of 1-4x10³ cells/well. After attachment, but prior to confluence (24-72h), the media was replaced with Osteogenic Differentiation Medium alone or with parathion, paraoxon, or vehicle (EtOH). This medium was replaced every 3-4d for 7-21d. Cells were fixed with absolute EtOH or 4% paraformaldehyde (PFA) for 20min then stained using either Alizarin Red or the OsteoImage[™] Mineralization Assay and measured using a microplate fluorescence spectrophotometer. Results were expressed as RFU.

Determination of Potential AChE Binding Partners in MSCs: The Pierce[™] Pull-Down PolyHis Protein:Protein Interaction Kit (Thermo Scientific) was used according to the manufacturer's recommended protocol. Briefly, previously prepared His-tagged AChE protein was incubated with MSC lysates for 1h at room temperature. These mixtures were placed on filter columns, washed, and protein eluted (column flow-through was kept from each step for analysis). The eluted protein and the initial flow-through were then prepared for SDS-PAGE and bands visualized using SimplyBlue[™] Safe Stain (Life Technologies). Band preparation for identification by mass spectrometry was completed using the In-Gel Tryptic Digestion Kit (Thermo Scientific) with destaining.

Confirmation of binding partner by co-immunoprecipitation (Co-IP): The immunoprecipitation protocol was adapted from the Protein A/G PLUS Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology) specification sheet. MSCs were lysed, collected, and assayed for protein concentration as described above (3.8). Lysate samples were diluted in 1X RIPA to within the concentration range suggested in the protocol. Lysates were pre-cleared with protein A/G-agarose beads (Santa Cruz Biotechnology) for 30min at 4°C with rotation, followed by centrifugation and supernatant collection. Anti-vinculin antibodies (Santa Cruz Biotechnology) were added to the supernatant followed by overnight incubation at 4°C with rotation. A/G-agarose beads were added to the tube again with overnight incubation at 4°C with rotation. Bead pellets were then washed several times with PBS, resuspended in sample buffer, prepared for SDS-PAGE, and processed for immunoblotting with vinculin or AChE.

Statistical Analysis: Two-way ANOVA were used to compare the mean responses among experimental and control groups. The Dunnett and Scheffe F-test was used to determine between which groups significant differences existed. A p-value of <0.05 was considered significant for all experiments.

INTRODUCTION

Figure 1

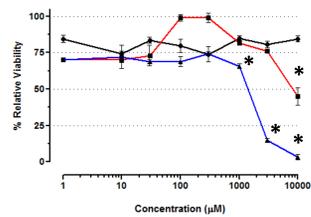


Figure 1: Effects of parathion and paraoxon on the viability of cultured human MSCs. Human MSCs were exposed to increasing concentrations of parathion (red, ■), paraoxon (blue, ▲), or equivalent amounts of vehicle control (EtOH, black, ●) for 48h, after which they were assayed for viability. The results are reported as mean ± SEM of % Relative Viability; n ≥ 4 for each condition tested. *p ≤ 0.05 versus vehicle control.

Figure 4

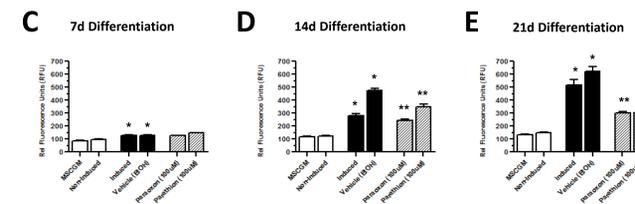
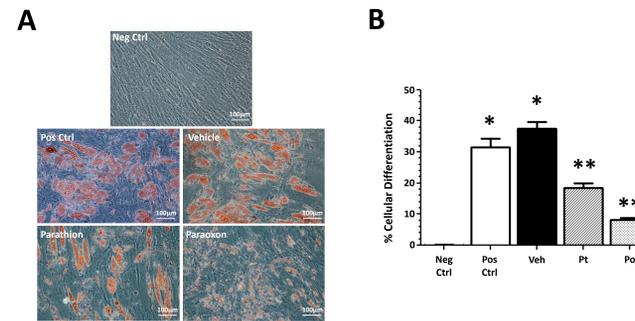


Figure 4: Human MSC adipogenic differentiation. A: Representative phase contrast images of Oil Red O stained adipogenic differentiated MSCs. Bar = 100µm. B: Analysis of photomicrographs of adipogenic differentiation using Image J software. Vertical bars represent the mean ± SEM of % Cellular Differentiation. C-E: Quantitative graph of relative fluorescence units (RFU) for each adipogenic differentiation condition. MSCs were exposed to adipogenic differentiation media for (C) 7, (D) 14, or (E) 21d and then evaluated for differentiation using the fluorescence-based AdipoRed Assay Reagent. The results are reported as mean ± SEM of RFU. Open bars represent negative control conditions; filled bars represent positive control conditions; cross-hatched bars represent experimental conditions. n = 16 for each condition. B-E: *p < 0.05 versus non-induced control; **p < 0.05 versus vehicle control.

Figure 2

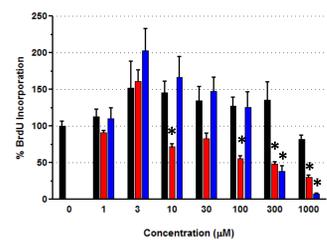


Figure 2: Effects of parathion and paraoxon on the proliferative ability of cultured human MSCs. Human MSCs were exposed to increasing concentrations of parathion (red), paraoxon (blue), or equivalent amounts of vehicle control (EtOH, black) for 48h. The cells were then assayed for cellular growth. The results are reported as mean ± SEM of % BrdU Incorporation; n ≥ 4 for each condition tested. *p ≤ 0.05 versus vehicle control.

Figure 5

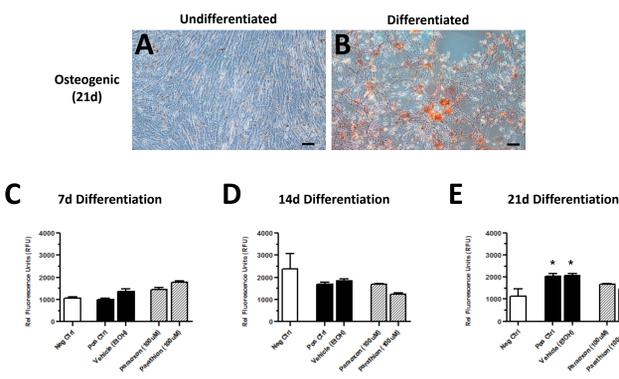


Figure 5: Evaluation of OPPs on MSC osteogenic differentiation. A-B: Representative phase contrast images of Alizarin Red stained (A) undifferentiated or (B) osteogenic differentiated MSCs. Bar = 100µm. C-E: Quantitative graph of RFU for each osteogenic differentiation condition. MSCs were exposed to osteogenic differentiation media for (C) 7, (D) 14, or (E) 21d and then evaluated using the OsteoImage Mineralization Assay. The results are reported as mean ± SEM of RFU. Open bars represent negative control conditions; filled bars represent positive control conditions; cross-hatched bars represent experimental conditions. n ≥ 16 for each condition. *p < 0.05 versus negative control; **p < 0.05 versus vehicle control.

Figure 3

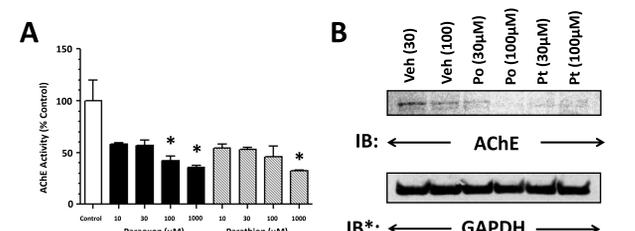


Figure 3: Effects of parathion and paraoxon on the activity and expression of AChE in human MSCs. A: MSCs were exposed to increasing concentrations of paraoxon (filled), parathion (cross-hatched), or media alone (open) for 24h. The cells were then assayed for AChE activity. Vertical bars represent mean ± SEM of AChE Activity (% Control); n ≥ 2 for each condition tested. *p ≤ 0.05 versus media control. B: Human MSCs were exposed to paraoxon, parathion, or vehicle control (EtOH) for 24h and blotted for AChE. IB: immunoblot. IB*: immunoblot of housekeeping gene product. The blot is representative of 3 independent experiments.

Figure 6

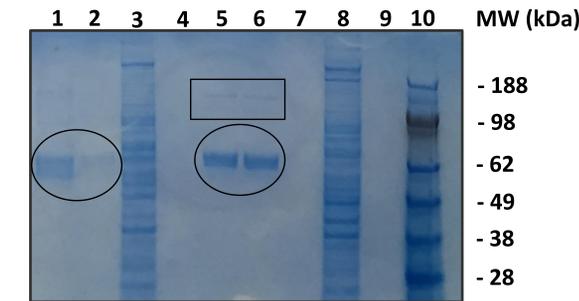


Figure 6: Identification of potential AChE binding partners in human MSCs. Human MSCs were lysed and combined with His-tagged human AChE protein using the Pierce[™] Pull-Down PolyHis Protein:Protein Interaction Kit as described in Materials and Methods. The resultant lysates were resolved by SDS-PAGE and stained with SimplyBlue stain to identify proteins. Mass spectrometry was then used to identify vinculin as a potential AChE binding partner. Ovals: AChE; Rectangle: Potential AChE Binding Partners (vinculin).

Figure 7

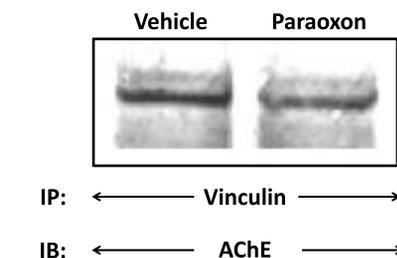


Figure 7: Association of AChE with the focal adhesion protein vinculin in MSCs. Human MSCs were exposed to either paraoxon or equivalent amounts of vehicle control (EtOH) for 24h. The MSCs were lysed and immunoprecipitated with anti-vinculin antibodies, resolved by SDS-PAGE, and transferred to PVDF membranes. The membranes were then incubated with anti-AChE antibodies followed by AP-conjugated secondary antibodies and then developed. Blot shown is representative of 3 independent experiments.

CONCLUSIONS

- Parathion/paraoxon reduce cellular viability of hMSCs
 - IC₅₀s: Paraoxon – 2000µM; Parathion ≥ 10000µM
- Parathion/paraoxon reduce the proliferative potential of hMSCs
 - Paraoxon ≥ 300µM; Parathion ≥ 100µM
- Parathion/paraoxon reduced both AChE activity and protein levels
- Parathion/paraoxon reduced the ability of hMSCs to differentiate into both adipocytes and osteoblasts.
- AChE binds to the focal adhesion protein, vinculin, in hMSCs. This interaction may be key to regulating hMSC proliferation and differentiation.

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