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Effects of organophosphates on the regulation of mesenchymal stem cell proliferation and differentiation

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent cells located within various adult tissues. Recent literature has reported that human bone marrow-derived MSCs express active acetylcholinesterase (AChE) and that disruption of AChE activity by organophosphate (OP) chemicals decreases the ability of MSCs to differentiate into osteoblasts. The potential role of AChE in regulating MSC proliferation and differentiation is currently unknown. In the present study, we demonstrate that MSCs exposed to OPs have both decreased AChE activity and abundance. In addition, exposure to these OPs induced cellular death while decreasing cellular proliferation. Exposures to these compounds also reduced the adipogenic/osteogenic differentiation potentials of the MSCs. To elucidate the possible role of AChE in MSCs signaling following OP exposure, we captured potential AChE binding partners by performing polyhistidine (His_8)-tagged AChE pulldowns, followed by protein identification using liquid chromatography mass spectrometry (LC-MS). Using this method, we determined that the focal adhesion protein, vinculin, is a potential binding partner with AChE in MSCs and these initial findings were confirmed with follow-up co-immunoprecipitation experiments. Identifying AChE binding partners helps to determine potential pathways associated with MSC proliferation and differentiation, and this understanding could lead to the development of future MSC-based tissue repair therapies.

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1. Introduction

Organophosphate (OP) compounds were initially developed as pesticides to control insects, but more toxic versions of these compounds have been developed and used as chemical warfare agents (CWAs) (e.g. sarin, soman, and VX) [1]. This class of chemicals typically acts by impacting the nervous system through the disruption of the normal action of the essential enzyme acetylcholinesterase (AChE) [2]. The main function of AChE is to terminate neural signal transmission through the degradation of the neural transmitter acetylcholine. When this normal cycle of acetylcholine is interrupted by OP compounds, acetylcholine accumulates in the neural synapse and induces cholinergic hyper-stimulation [3]; in acute situations, this hyper-stimulation can lead to respiratory failure in humans [4]. Although AChE is conventionally known for this signaling role in neurons, it has been speculated that AChE may play a role in non-neural signaling [5,6]. In fact, AChE has been shown to be expressed in several non-neuronal cell types including fibroblasts [7], osteoblasts [8–10], hematopoietic cells [11], and endothelial cells [12]. Research has suggested that, in these non-neuronal cells, AChE may be involved in the regulation of cellular adhesion [6,13].

Mesenchymal stem cells (MSCs) can be isolated from various adult tissues and play a significant role in tissue maintenance and repair [14,15]. Also known as marrow stromal cells, bone marrow-derived MSCs were initially described in 1968 by Friedenstein and colleagues [16] for their characteristic ability to attach to tissue culture plastic and having a fibroblast-like morphology. Since their initial discovery in bone marrow, MSCs have been isolated from

Abbreviations: AChE, Acetylcholinesterase; AP, alkaline phosphatase; CWAs, chemical warfare agents; Co-IP, Co-Immunoprecipitation; EtOH, ethanol; FA, focal adhesion; LC-MS, liquid chromatography mass spectrometry; MSC, mesenchymal stem cells; MSCGM, Mesenchymal Stem Cell Growth Medium; MSCGS, Mesenchymal Stem Cell Growth Supplement; MW, molecular weight; OP, organophosphate; PFA, paraformaldehyde; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay buffer; RFU, relative fluorescence units; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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several other tissues including umbilical cord, Wharton's jelly of the umbilical cord, placenta, adipose tissue, dental pulp, as well as from the lungs of lung transplant patients [17–24]. MSCs are capable of self-renewal and can be maintained in a multipotent state *in vitro* [14]. Overall, the MSCs are at least in part responsible for maintaining homeostasis in adult tissues [25].

A limited number of studies have demonstrated that MSCs express active AChE [26,27]. The first of these two studies also demonstrated that inhibition of AChE activity levels by either carbofuran or chloropyrifos affects the ability of MSCs to differentiate into osteoblasts [27]. A more recent study demonstrated that a mixture of low-level pesticides, including OPs, inhibited the ability of MSCs to differentiate into osteoblasts [28]. These results suggest a potential link between AChE activity and the differentiation capacity of MSCs. In addition to enzymatic activity, AChE has been shown to be involved in the regulation of cell adhesion and motility in other cell types. In fact, AChE seems to play a role in normal bone development [8,9,29] and exposure to OPs has been shown to alter normal bone development [30]. It is also interesting to note that AChE has been shown to be involved in the regulation of cell adhesion and motility in other cell types [6,8,9,13,31–33]. Since cell adhesion and motility are essential to MSC differentiation, it is possible that AChE is playing a regulatory role in this pathway.

In this study, we demonstrated that the OP pesticide parathion and its metabolite paraoxon reduce the viability, proliferation capacity, and differentiation potential of bone marrow-derived MSCs; these reductions are also associated with reduced AChE protein expression and activity levels in these cells. Paraoxon induced a dose-dependent reduction in cellular viability following a 48 h treatment while parathion induced cell death at concentrations >3000 μM. Following 48 h treatment with parathion/paraoxon at concentrations that did not induce cellular death, normal cellular proliferation was interrupted. Treatment of MSCs with these chemicals also induced a concentration-dependent decrease in both AChE activity and protein levels. Also, parathion or paraoxon treatment reduced the ability of MSCs to differentiate into either adipocytes or osteoblasts. Finally, by LC-MS analysis and immunoprecipitation, we were able to demonstrate that AChE interacts with the focal adhesion (FA) protein vinculin. Collectively, these data suggests that AChE is an important signaling element in MSC proliferation and differentiation.

2. Materials and methods

2.1. Experimental chemicals

Parathion and paraoxon were purchased from ULTRA Scientific (N. Kingstown, RI). Stock solutions were prepared in 100% denatured ethanol (EtOH) and stored at 4 °C until used as previously described [34].

2.2. Human MSC culture

Primary human bone marrow-derived MSCs were obtained from Lonza (Walkersville, MD) and cultured in Mesenchymal Stem Cell Growth Medium (MSCGM) supplemented with Mesenchymal Stem Cell Growth Supplement (MSCGS), L-glutamine, gentamicin, amphotericin-B (all supplements from Lonza) as previously described [35,36]. Culture medium was refreshed every 48–72 h and cells were subcultured upon confluency using the manufacturer's recommended protocol. Only MSCs from passages 4–8 were used in these studies.

2.3. Parathion/paraoxon toxicity studies

To evaluate the toxicity of parathion and paraoxon on MSCs, 1×10^4 MSCs were plated in each of the wells of 96-well tissue culture plates, allowed to attach for 24 h and then exposed to increasing concentrations (1, 10, 30, 100, 300, 1000, 3000, 10000 μM) of parathion, paraoxon, or equivalent volumes of vehicle control (EtOH) for 24 or 48 h. Following these exposures, an MTT Cell Viability Assay (Roche Applied Science; Indianapolis, IN) was performed as previously described [34]. Results were read on a SpectraMax® Plate Reader (Molecular Devices, LLC; Sunnyvale, CA) and expressed as percent relative viability.

2.4. MSC proliferation studies

To evaluate the effects of parathion/paraoxon on MSC proliferation, 5×10^3 MSCs were plated per well in 96-well tissue culture plates, allowed to attach for 24 h, then exposed to increasing concentrations (30, 100, 300, 1000 μM) of parathion, paraoxon, or equivalent volumes of vehicle control (EtOH) for 48 h. These specific OP concentrations were chosen because these were the highest concentrations examined in the viability assay that caused minimal or no loss of cellular viability during the 48 h exposure studies. MSC proliferation was subsequently evaluated using the BrdU Cell Proliferation Assay (Roche Applied Science) according to the manufacturer's instructions. Plates were then read on a SpectraMax® Plate Reader and results were expressed as percent BrdU incorporation.

2.5. Determination of AChE activity

AChE activity within the MSCs was measured using the colorimetric Acetylcholinesterase Assay Kit (Abcam; Cambridge, MA). Prior to the AChE activity assay MSCs were plated in a 96-well tissue culture plates and exposed to increasing concentrations of parathion or paraoxon (10, 30, 100, 1000 μM), or equivalent volumes of vehicle control (EtOH) for 24 h. These OP concentrations were chosen based on the results of the MSC viability studies. Following exposure, the media was removed and the MSCs were lysed as directed by the manufacturer's protocol using the lysis buffer included in the assay kit. An acetylthiocholine reaction mixture (included in the assay kit) was added to each well and incubated at room temperature in the dark for 0.5 h. The plate was then read at OD_{410} on a SpectraMax® Plate Reader and AChE activity was reported as a percentage of AChE activity exposed only to vehicle control (EtOH).

2.6. Determination of AChE protein expression in MSCs

Protein expression levels of AChE in MSCs were determined through western blot analysis. MSCs were exposed to parathion or paraoxon (30 or 100 μM), or equivalent amounts of vehicle control (EtOH) for 24 h. These OP concentrations were based on the results of the experiments stated above. Following exposure, the MSCs were lysed and collected in 1X radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Inc.; Danvers, MA), centrifuged (12,000 rpm × 10 min at 4 °C), and the supernatants assayed for protein concentration with the Pierce™ 660 nm Protein Assay (Thermo Fisher Scientific; Rockford, IL). Proteins were resolved using the 4–12% gradient Bolt™ Bis-Tris Plus Gel according to the manufacturer's instructions (Life Technologies; Grand Island, NY) and transferred onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes (Life Technologies) using the iBlot® 7-Minute Blotting System (Life Technologies). The membranes were blocked with iBlot® blocking solution, incubated with rabbit