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Mesenchymal Stem Cells From Different Murine Tissues Have Differential Capacity to Metabolize Extracellular Nucleotides

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ABSTRACT

Mesenchymal stem cells (MSCs) have shown a great potential for cell-based therapy and many different therapeutic purposes. Despite the recent advances in the knowledge of MSCs biology, their biochemical and molecular properties are still poorly defined. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-5′-nucleotidase (eNT/CD73) are widely expressed enzymes that hydrolyze extracellular nucleotides, generating an important cellular signaling cascade. Currently, studies have evidenced the relationship between the purinergic system and the development, maintenance, and differentiation of stem cells. The objective of this study is to identify the NTPDases and eNT/CD73 and compare the levels of nucleotide hydrolysis on MSCs isolated from different murine tissues (bone marrow, lung, vena cava, kidney, pancreas, spleen, skin, and adipose tissue). MSCs from all tissues investigated expressed the ectoenzymes at different levels. In MSCs from pancreas and adipose tissue, the hydrolysis of triphosphonucleosides was significantly higher when compared to the other cells. The diphosphonucleosides were hydrolyzed at a higher rate by MSC from pancreas when compared to MSC from other tissues. The differential nucleotide hydrolysis activity and enzyme expression in these cells suggests that MSCs play different roles in regulating the purinergic system in these tissues. Overall MSCs are an attractive adult-derived cell population for therapies, however, the fact that ecto-nucleotide metabolism can affect the microenvironment, modulating important events, such as immune response, makes the assessment of this metabolism an important part of the characterization of MSCs to be applied therapeutically. J. Cell. Biochem. 115: 1673–1682, 2014.

KEY WORDS: KEY WORDS: PURINERGIC SIGNALING; ECTONUCLEOTIDASES; MSC; E-NTPDASES; CD73

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Mesenchymal stem cells (MSCs) were first identified more than 30 years ago by Friedenstein et al. [1976]. Ever since, the number of preclinical and clinical studies that utilize these cells has greatly increased. MSCs have self-renewal properties and the capacity to differentiate into multiple lineages such as osteocytes, adipocytes, and chondrocytes, therefore, being considered the adult stem cell type of greatest plasticity [Chagastelles et al., 2010]. Furthermore, they can be isolated from virtually any organ or tissue, such as brain, heart, lung, bone morrow, kidney, and spleen [da Silva Meirelles et al., 2006; Murrell et al., 2013]. MSCs are also easily enriched by isolation of cells with fibroblast-like morphology via their preferential attachment to plastic tissue culture dishes. These characteristics have contributed to make these cells an ideal source of stem cells for therapy, regenerative medicine, and transplantation [Chamberlain et al., 2007].

MSCs have been isolated from different sources, and although the cells from different tissues are relatively similar, each type has been reported to vary in their properties, such as proliferative and developmental potential [da Silva Meirelles et al., 2008; Sung et al., 2008]. Moreover, the expression of some surface markers is variable among the different MSCs populations originated from distinct tissues [da Silva Meirelles et al., 2006; Noel et al., 2008].

The importance of extracellular nucleotide hydrolysis for homeostasis in several tissues has been extensively reported. Extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) constitute an important and ubiquitous class of extracellular molecules that regulate a variety of pathophysiological functions, including cell death, proliferation and differentiation by activating purinergic receptors [Burnstock, 2009]. Nucleotide mediated signaling is controlled by a enzymatic cascade, which includes the members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, (ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, ecto-5′-nucleotidase/CD73 (e5NT/CD73), and ecto-alkaline phosphatases, which can efficiently hydrolyze ATP to adenosine in the extracellular space.

In mammals, eight different *ENTPD* genes encode members of the NTPDase protein family. Four of them are typical cell surface-located enzymes with a catalytic site facing the extracellular compartment (NTPDase1, 2, 3, 8). NTPDases 5 and 6 exhibit intracellular localization and undergo secretion after heterologous expression. NTPDases 4 and 7 are entirely intracellularly located, anchored to the membranes of organelles. These enzymes can be differentiated according to substrate preference, divalent cation usage, and product formation. The AMP produced in the extracellular space is hydrolyzed to adenosine by the action of eNT/CD73. This enzyme is linked to the plasma membrane with its catalytic site exposed to the extracellular space, and is the major contributor to the pathway that completely hydrolyses extracellular ATP to adenosine [Zimmermann et al., 2012].

Extracellular ATP has been identified as an important molecule released from human MSCs with capacity to modulate their proliferation rate and their cell fate. Coppi et al. demonstrated that ATP spontaneously released from bone marrow-derived human MSCs (hMSCs), at early stages of culture (P0–P5), is able to decrease cell proliferation. The results showed that the concentration of extracellular ATP measured in the medium containing hMSCs was eightfold higher than that measured in the medium without cells. This raises the possibility that this nucleotide can act as one of the factors determining the hMSCs physiology [Coppi et al., 2007]. In another work, ATP has been appointed as an essential molecule to astrocyte-mediated proliferation of neural stem cells (NSC) in vitro. Astrocytes unable to release ATP led to a failure in NSC proliferation in the adult hippocampus. On the other hand, the reestablishment of normal levels of ATP via exogenous administration reversed this effect [Cao et al., 2013]. Another study suggested that purine nucleotides can act as modulators of the proliferation of preadipocytes, showing that low concentrations of ATP (100 μM) is able to stimulate the proliferation of brown fat preadipocytes from rat, while higher concentrations of adenosine (250–500 μM) inhibited this proliferation [Wilson et al., 1999]. Recently, Ferrari et al. [2011], demonstrated that bone marrow-derived hMSCs undergo a decrease in their proliferation rate after stimulation with ATP, whereas their migration rate was markedly increased, thereby increasing the homing ability of hMSCs to the bone marrow of immunodeficient mice when cells were pretreated with ATP. ATP seems also to be involved in the control of expression of several genes related with adipogenic and osteoblastic differentiation in MSCs. ATP, through specific receptors, stimulates adipogenic phenotype in bone marrow-derived hMSCs by increasing lipid accumulation and expression levels of PPARγ (peroxisome proliferator-activated receptor-gamma), a receptor that has been implicated in adipogenesis processes, while the ATP degradation product, adenosine, induces osteogenic differentiation [Ciciarello et al., 2013]. In addition, several P2 receptors change their expression during both, adipogenic and osteogenic differentiation of adipose derived hMSCs and dental follicle cells [Zippel et al., 2012], reinforcing the idea of nucleotides signaling in stem cell differentiation. Sak et al. [2003] reported that P2Y receptors have important roles in the hematopoietic cells differentiation, besides showing changes in the purinergic receptors expression during the maturation process of these cells. Rat adipose derived MSCs express P2X3, P2X4, and P2X7 purinoceptors and, interestingly, P2X4 and P2X7 are upregulated in MSCs differentiated to a glial phenotype [Faroni et al., 2013]. In mice bone marrow-derived MSCs, the lack of P2Y13 receptor caused a decrease in bone restoration and a reduction in the number of both osteoblasts and osteoclasts on the bone surfaces. In contrast, P2Y13 deficient cells with a adipogenic phenotype, had an increase of number of adipocyte generated and higher levels of adipogenic gene expression than those obtained in MSCs with functional P2Y13 [Biver et al., 2013]. Lin et al. showed that the neural progenitor cells are the source of local ATP and the neural differentiation in vitro is accompanied by a decrease in ATP release and by loss of functional P2Y receptors. In contrast, receptor antagonists suppress proliferation and allow the cellular differentiation into neurons and glia in vitro [Lin et al., 2007]. Adenosine also can be an important regulator of cell differentiation through the activation of subtype-specific receptors. For example, in rat and human bone marrow-derived MSCs, the expression of A2b receptor is upregulated during osteoblast differentiation [Costa et al., 2011; Gharibi et al., 2011]. On the other hand, adipogenic differentiation is accompanied by increases in A1 and A2a expression levels [Gharibi et al., 2011].

Indeed, the overexpression of A1 receptor in an osteoblast precursor cell line promotes differentiation to adipogenic instead of oesteoblastic phenotype, while de overexpression of the A2b receptor inhibits adipogenic and favors osteoblastic differentiation [Gharibi et al., 2012].

Members of ectonucleotidases families as well as purinergic receptors are expressed in different types of stem cells [Shukla et al., 2005; Mishra et al., 2006; Lin et al., 2007]. The extracellular nucleoside triphosphate-hydrolyzing enzyme, NTPDase2, appears to be highly expressed by stem/progenitor cells in neurogenic regions of the adult murine brain and seems to contribute to the control of adult neurogenesis [Shukla et al., 2005]. Additionally, NTPDase 1 and 8 are responsible for the metabolism of ATP in murine bone marrow-derived MSCs differentiated in osteoblasts, whereas NTPDase 3 is involved with its metabolism in undifferentiated MSCs [Roszek et al., 2013]. These results besides contributing to better understanding of interaction between purinergic system and stem cells, also rise the possibility to use these enzymes as markers for differentiation in MSCs.

Despite this considerable literature pointing to the importance of purinergic signaling in stem cells [Boskey et al., 1994; Agresti et al., 2005; Mishra et al., 2006, Coppi et al., 2007; Grimm et al., 2010; Streitova et al., 2010], the presence of ectonucleotidases and differences in the profile of nucleotides hydrolysis by MSC isolated from different tissues have not yet been reported. Therefore, the present study describes the extracellular nucleotide metabolism in MSCs originated from different murine tissues in order to contribute to a better understanding of the workings of the purinergic system in these cells and in their microenvironment.

MATERIALS AND METHODS

ETHICS

All the experimental procedures were performed according to institutional guidelines and were approved by the Animal Use Ethic committee (CEUA) from UFCSPA at number 509-07.

CELL CULTURE

MSCs from bone marrow, lung, vena cava, kidney, pancreas, spleen, skin, and adipose tissue were isolated from adult BALB/c strain mice (6–8 weeks old). Details on the isolation of MSCs have been described previously [da Silva Meirelles et al., 2006]. Briefly, organs and tissues were collected, rinsed in HB-CMF-HBSS, transferred to a Petri dish and cut into small pieces and subsequently digested with collagenase type I (0.5 mg/ml in DMEM/10 mM HEPES) for 30 min at 37°C. After collagenase digestion, the supernatant was transferred to a new tube and centrifuged at 400*g* for 10 min at room temperature (RT), the pellets were resuspended in complete medium and seeded in six-well dishes. MSCs cultures were grown in culture flasks and maintained in 1% low glucose Dulbecco's modified Eagle's medium (DMEM), containing 8.39 mM HEPES (pH 7.4), 23.8 mM $NaHCO_3$, 0.1% fungizone, 0.5 U/ml penicillin/streptomycin (Gibco BRL, Grand Island, NY) and supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL). Cells were kept at a temperature of 37°C, humidity of 95%/5% CO_2 in air. Cultured MSCs were tested for the ability to differentiate into adipogenic and osteogenic cell lineage as described by da Silva Meirelles et al. [2006].

PHENOTYPE IDENTIFICATION

To confirm that MSCs maintain their phenotypic characteristics in culture, undifferentiated MSCs from spleen, adipose tissue, lung, bone marrow, kidney, skin, pancreas, and vena cava were subjected to flow cytometry analysis. The cells were trypsinized, centrifuged, and incubated for 30 min at 4°C with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against murine CD31, CD45, and CD90.2 (Pharmingen BD, San Diego, CA and eBioscience, San Diego, CA). Excess of antibody was removed by washing in PBS (phosphate buffered saline). The cells were analyzed using a FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA) with the CellQuest software. At least 10,000 events were collected. The WinMDI 2.8 software was used for building the histograms [da Silva Meirelles et al., 2006].

NUCLEOTIDE HYDROLYSIS ON CELLS SURFACE

Twenty four-multiwell plates containing MSCs from all tissues analyzed were washed thrice with a phosphate-free incubation medium in the absence of nucleotides. The reaction was started by the addition of 200 μl of incubation medium containing 2 mM $CaCl_2$, plus 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES (pH 7.4), and 1 mM of the ATP or ADP as substrates, at 37°C [Wink et al., 2003b]. The nucleotide concentrations and the incubation time were chosen to assure the linearity of the reaction. After 10 min of incubation for ATP and ADP, the reaction was stopped by taking an aliquot of the incubation medium and transferring to a tube containing TCA (5% w/v) previously placed on ice. The inorganic phosphate (Pi) released was measured by the malachite green method [Chan et al., 1986], using KH_2PO_4 as a Pi standard. The non-enzymatic Pi released from the nucleotide into the assay medium without cells was subtracted from the total Pi released during the incubation, giving net values for enzymatic activity. Specific activity was expressed as nmol Pi released/min/mg of protein.

ECTO-5′-NUCLEOTIDASE/CD73 ASSAY

Ecto-5′-nucleotidase activity was determined as described above for the analysis of extracellular ATP and ADP metabolism, except that was used an incubation medium containing 2 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, pH 7.4, and 1 mM of AMP as substrate, at 37°C [Wink et al., 2003b] . Similarly as described above, the nucleotide concentrations and the incubation time were chosen to assure the linearity of the reaction. Then, 30 min was chosen for the incubation of AMP and 1 mM of this nucleotide was used as substrate. Specific activity is expressed as nmol Pi/min/mg of protein [Chan et al., 1986].

ALKALINE PHOSPHATASE ASSAY

The extracellular phosphatase activity was determined in the same medium described for AMP hydrolysis except that 1 mM of glucose-6-phosphate or β-glycerophosphate was used as substrate [Millan, 2006]. Alternatively, the AMP hydrolysis was monitored in the presence of 1 mM levamisole, a specific inhibitor of tissue non-specific alkaline phosphatase [Scheibe et al., 2000].

PROTEIN DETERMINATION

Cells in the 24 multiwell plates were dried and solubilized with 100 μl of NaOH 1 N and frozen overnight. An aliquot was then taken and the protein was measured by the Comassie blue method [Bradford, 1976], using bovine serum albumin as standard.

RNA EXTRACTION, cDNA SYNTHESIS, AND RT-PCR ANALYSIS

Total RNA from MSCs cultures was isolated with Trizol LS reagent (Life Technologies, Carlsbad, CA) in accordance with the manufacturer's instructions. The cDNA species were synthesized with M-MLV reverse transcriptase (Promega, Madison, WI) from up to 5 μg of total RNA in a final volume of 25 μl with a oligo (dT) primer in accordance with the manufacturer's instructions. cDNA reactions were performed for 50 min at 37°C and stopped by freezing at 4°C. One microliter of the RT reaction mix was used for PCR in a total volume of 25 μl, which included 0.5 μM of each specific primer to NTPDases 1–3, 5, and 6, eNT/CD73 (Table I), 50 μM dNTPs, and 1 U of Taq polymerase (Promega) in the supplied reaction buffer. The PCR cycling conditions were as follows for the enzymes semiquantitative expression analyses: 1 min at 95°C, 1 min at 94°C, 1 min at annealing temperature, 1 min at 72°C. PCR reactions were carried out for 35 (to enzymes genes) and included a final 10 min extension at 72°C. Ten microliters of the PCR reaction were analyzed on a 2.0% agarose gel containing *SYBRGold* (Gibco BRL) and visualized under ultraviolet light. As a control for cDNA synthesis, β-actin PCR was performed. Negative controls were performed by substituting DNAse/RNAse-free distilled water for the templates in each PCR reaction.

REAL-TIME PCR ANALYSIS

Real-time PCR amplification was carried out using specific primer pairs to NTPDase 1–3, 5, 6 and eNT/CD73 (Table I). Real-time PCRs were carried out in an Applied-Biosystem StepOnePlus™ Real-Time PCR cycler and done in duplicate. Reaction settings were composed of an initial enzyme activation step of 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C for data acquisition; samples were kept for 15 s at 95°C and then heated from 70 to 99°C, during 1 min and 15 s, respectively, with a ramp of 0.1°C/s to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were made in a 18 μl final volume composed of 9 μl of Fast SYBRgreen master mix (Applied Biosystems, Foster City, CA), 1 μl of 10 μM primer pairs, 7 μl of water, and 1 μl cDNA. All results were analyzed by the $2^{-\Delta\Delta CT}$ method, using the β-actin as normalizator and adipose tissue as a regulator in order to make comparisons [Livak and Schmittgen, 2001]. The real-time PCR products were separated by 2.0% agarose gel containing *SYBRGold* (Gibco BRL) and visualized under ultraviolet light.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard error of mean (SEM) and were subjected to one-way analysis of variance (ANOVA) followed by Tukey posthoc test (for multiple comparisons) using the statistical program, SPSS 10.0 for Windows. Differences between mean values were considered significant when $P < 0.05$.

RESULTS

MSC CHARACTERIZATION

Isolated MSCs from all tissues adhered to plastic and showed a fibroblast-like morphology as can be expected for MSCs (Fig. 1a). Functional assays to confirm the MSC identity of the populations studied were performed to evaluate the capacity of the cells to differentiate. When subjected to osteogenic or adipogenic differentiation conditions, the MSC populations stem cell characteristics by depositing a calcium-rich mineralized matrix as evidenced by Alizarin Red S staining, or by acquiring intracellular lipid droplets, evidenced by Oil Red O staining (Fig. 1b,c). Data from undifferentiated and differentiated adipose-derived MSCs are shown, but all tissues had very similar capacity of differentiation.

The analysis of surface markers indicated that the MSC populations originating from different sources have a very similar immunophenotype. All the populations studied expressed CD90.2 (Thy1.2) as demonstrated by flow cytometry. The hematopoietic marker CD45 (leukocyte common antigen) was not expressed by MSCs. MSC cell populations were also negative for the endothelial marker CD31 (platelet endothelial cell adhesion molecule-1) (Fig. 2). All experiments were performed using cells between 4 and 15 passages.

ATP, ADP, AND AMP HYDROLYSIS

Substrate concentration and incubation time were previously determined in order to ensure the linearity of the reaction

TABLE I. Primer Sequences, Annealing Temperatures, and Fragment Size

	Primer sequence	T (°C)	Fragment size (bp)
NTPDase1 F	3′GAT CAT CAC TGG GCA GGA GGA AGG5′	60	543
NTPDase1 R	3′AAG ACA CCG TTG AAG GCA CAC TGG5′		
NTPDase2 F	3′GCT GGG TGG GCC GGT GGA TAC G5′	60	331
NTPDase2 R	3′ATT GAA GGC CCG GGG ACG CTG AC5′		
NTPDase3 F	3′TCT AGA GGT GCT CTG GCA GGA ATC AGT5′	60	267
NTPDase3 R	3′TCT AGA GGT GCT CTG GCA GGA ATC AGT5′		
NTPDase5 F	3′GGG ATC CTT TGA GAT GTT TAA CAG CAC T5′	60	210
NTPDase5 R	3′GAA TTC TTG GTT ACC ACC ATA CTG GTA5′		
NTPDase6 F	3′GAA TTC CTT GTC GGG GAT GAC TGT GTT5′	60	156
NTPDase6 R	3′ATC TGA GTG GAT CCT CCG CCC AA5′		
eNT/CD73 F	3′CCC GGG GGC CAC TAG CAC CTC A5′	60	403
eNT/CD73 R	3′GCC TGG ACC ACG GGA ACC TT5′		
β-Actin F	3′TAT GCC AAC ACA GTG CTG TCT GG5′	60	210
β-Actin R	3′TAC TCC TGC TTC CTG ATC CAC AT5′		

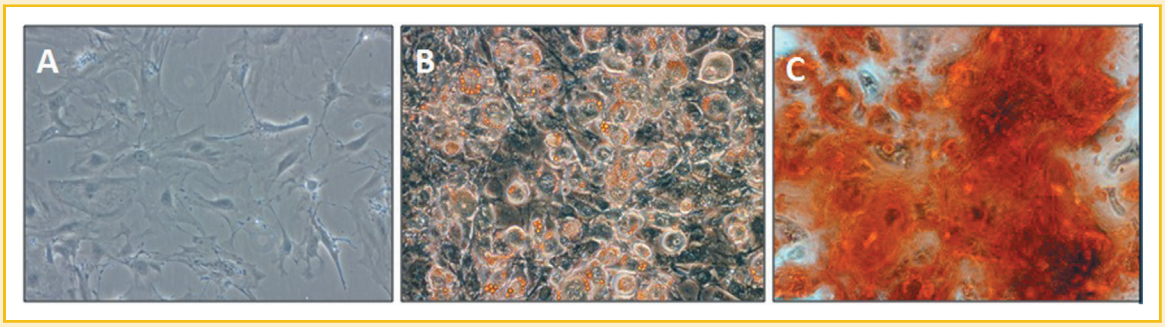

Fig. 1. Adipogenic and osteogenic induction of MSCs derived from adipose tissue. Adipose-derived MSC cultures at passage 5 were cultured in standard culture medium, osteogenic or adipogenic differentiation medium for up to 2 months as described in the Materials and Methods section. A: Phase-contrast micrographs of control MSC culture, using standard medium culture as described. B: Lipid vacuoles are stained orange with Oil Red O and (C) calcium deposited in the extracellular matrix is stained red by Alizarin Red S. Magnifications, 200×.

(data not shown). The experiments were performed with substrate concentrations of 1.0 mM. The incubation time of 30 min was used for AMP, glucose-6-phosphate, and β-glycerophosphate substrates, while the time chosen for ATP and ADP incubation was 10 min. Next, we analyzed the pattern of ATP, ADP, and AMP hydrolysis by the MSCs originated from eight organs: pancreas, adipose tissue, skin, vena cava, bone marrow, kidney, lung, and spleen. The extracellular hydrolysis of ATP by these cells was in the order of pancreas > adipose tissue > skin > vena cava > bone marrow > kidney > lung > spleen (Table II and Fig. 3a). There was no statistical difference between MSCs from kidney, bone marrow, spleen, vena cava, skin, and lung. Pancreas and adipose tissue-derived MSCs showed similar values of ATP hydrolysis and it is important to note that these values were significantly higher when compared with the other cell types. ADP hydrolysis was low in MSCs from lung, bone marrow, kidney, spleen, vena cava, and skin, while pancreas-derived MSCs had higher ADPase activity, differing from all other cell types (Table II and Fig. 3b).

The extracellular AMP metabolism of MSCs was also analyzed and bone marrow-derived MSCs showed values of hydrolysis significantly higher when compared with the other cell types (Table III and Fig. 4a).

ECTO-PHOSPHATASE ASSAY

The extracellular alkaline phosphatase activity was analyzed in the presence of the specific inhibitor levamisole in comparison with AMP hydrolysis in the absence of this inhibitor (Table III). The AMP hydrolysis in this condition was not detected in lung-derived MSC (Fig. 4a). Bone marrow-derived MSCs showed higher rate of AMP hydrolysis when compared with MSCs derived from pancreas, kidney, spleen, lung, vena cava, and skin. In the presence of the inhibitor of alkaline phosphatase, levamisole, the hydrolysis of AMP by bone marrow-derived MSCs was reduced approximately threefold, whereas in other cells the difference was not statistically significant. This result suggests that besides the presence of CD73, other enzymes, such as alkaline ecto-phosphatase, are enzymatically active on the surface of these cells.

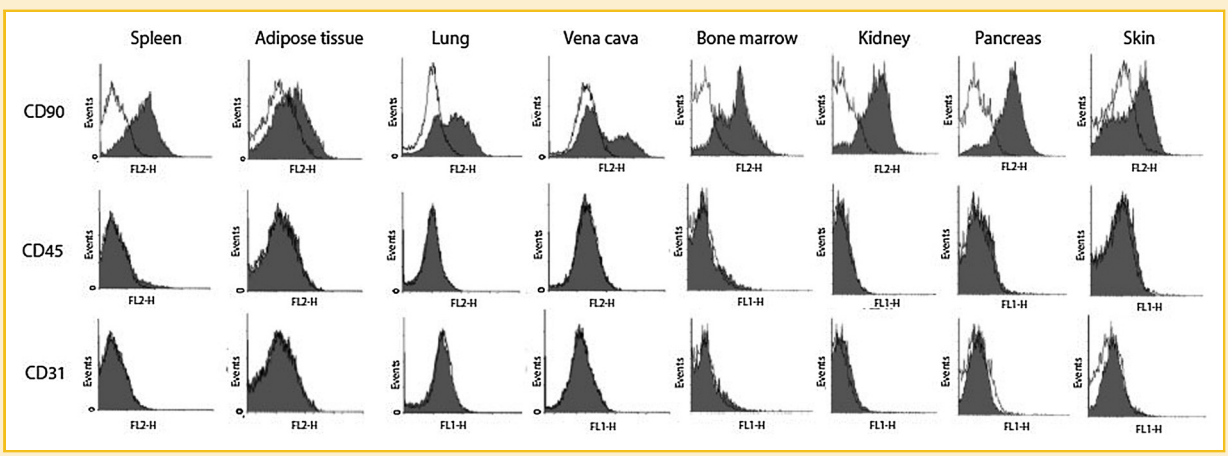

Fig. 2. Immunophenotypic profile of MSCs derived from different murine tissues. Flow cytometry histograms show the expression (shaded) of selected molecules (CD90.2, CD45, and CD31) by different MSC populations compared with controls (unshaded peaks).

TABLE II. Specific Activities for the ATP and ADP Hydrolysis

	Specific activities (nmol Pi/min/mg of protein)	
Cellular origin	ATP	ADP
Pancreas	22.32 ± 0.9	12.19 ± 0.21
Adipose tissue	18.76 ± 5.75	8.16 ± 3.01
Skin	10.08 ± 1.18	1.15 ± 1.15
Vena cava	5.07 ± 0.13	2.98 ± 0.22
Bone marrow	3.06 ± 1.21	5.07 ± 1.21
Kidney	2.27 ± 0.83	3.68 ± 2.33
Lung	2.0 ± 0.4	1.47 ± 0.47
Spleen	0.6 ± 0.6	1.98 ± 0.52

Results are expressed as mean ± standard error of mean (SEM) of at least three experiments for nucleotides hydrolysis.

TABLE III. Specific Activities for the AMP Hydrolysis in the Presence or Absence of Levamisole, the Specific Inhibitor of Alkaline Phosphatase

	Specific activities (nmol Pi/min/mg of protein)	
Cellular origin	AMP	AMP + levamisole
Bone marrow	10.22 ± 0.56	3.22 ± 0.44
Adipose tissue	6.47 ± 3.47	3.45 ± 2.55
Pancreas	2.45 ± 1.81	1.16 ± 0.24
Kidney	1.72 ± 0.55	2.46 ± 0.83
Spleen	1.41 ± 0.31	0.95 ± 0.05
Lung	1.01 ± 0.19	No detected
Vena cava	0.91 ± 0.29	0.88 ± 0.13
Skin	0.83 ± 0.57	0.91 ± 0.29

Results are expressed as mean ± standard error of mean (SEM) of at least three experiments for nucleotides hydrolysis.

Alkaline phosphatases are known to hydrolyze a variety of organic phosphates. To better investigate the presence of this ecto-enzyme in MSCs, their extracellular phosphatase activity was examined using glucose-6-phosphate and β-glycerophosphate as substrates (Table IV and Fig. 4b). The alkaline phosphatases appear to exert a considerable contribution on AMP hydrolysis by all MSCs studied. However, among the studied tissues, the MSCs from bone marrow presented the highest rates of hydrolysis for glucose-6-phosphate and β-glycerophosphate. These substrates were degraded with specific activities that were not significantly different when compared with the AMP hydrolysis (Fig. 4a,b). These data suggest the presence of an extracellular alkaline phosphatase activity in MSCs.

ECTONUCLEOTIDASES mRNA EXPRESSION

As ATP, ADP, and AMP can be metabolized by different members of the ectonucleotidases family and to better characterize the enzymes involved in extracellular nucleotide hydrolysis in MSCs, we investigated the mRNA expression of the main enzymes, *Entpd* 1, 2, 3, 5 e 6, and e5NT/CD73 in MSCs from adipose tissue, vena cava, kidney, bone marrow, lung, skin, spleen, and pancreas (Table V). All members of ectonucleotidase family analyzed were detected in MSCs, with *Entpd* 1 and 5 present in all tissues. Quantitative real-time RT-PCR analysis indicated that MSCs derived from lung, skin, and pancreas were the cells that express higher levels of mRNA of the CD73 and NTPDases (Fig. 5).

DISCUSSION

The understanding of the central proliferation and differentiation mechanisms of MSCs has attracted much attention in the last years.

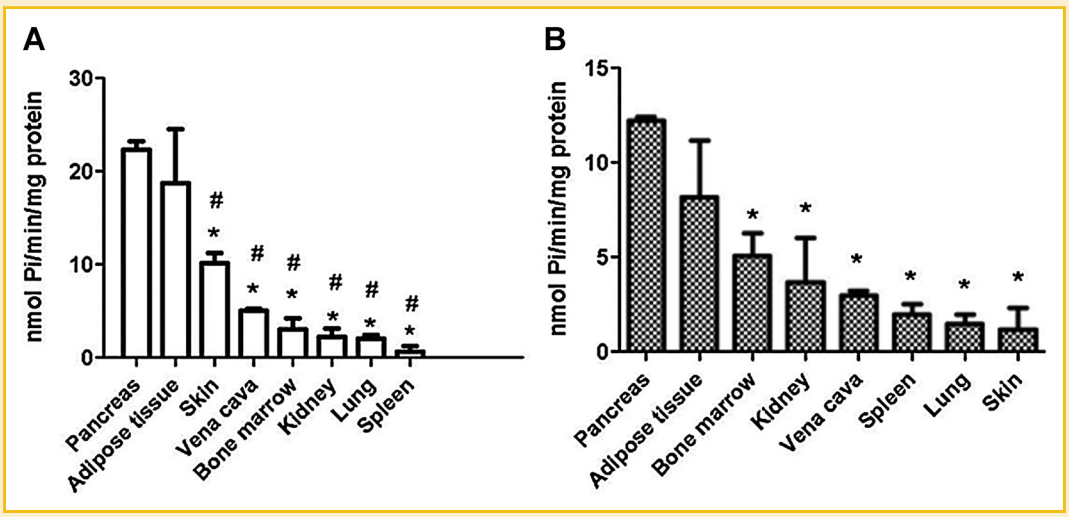

Fig. 3. ATP and ADP hydrolysis on the surface of MSCs from different murine tissues. Following confluence, MSCs from pancreas, adipose tissue, skin, bone marrow, vena cava, kidney, lung, and spleen were incubated with ATP (A) or ADP (B) as described in the Materials and Methods section. Specific activities were expressed as nmol Pi/min/mg of protein. Bars represent mean ± SEM of at least three independent experiments performed in triplicate. It was considered significance when $P < 0.05$. Data were analyzed statistically by one-way ANOVA followed by Tukey post-test. A: *Different from pancreas and #different from adipose tissue. B: *Different from pancreas.

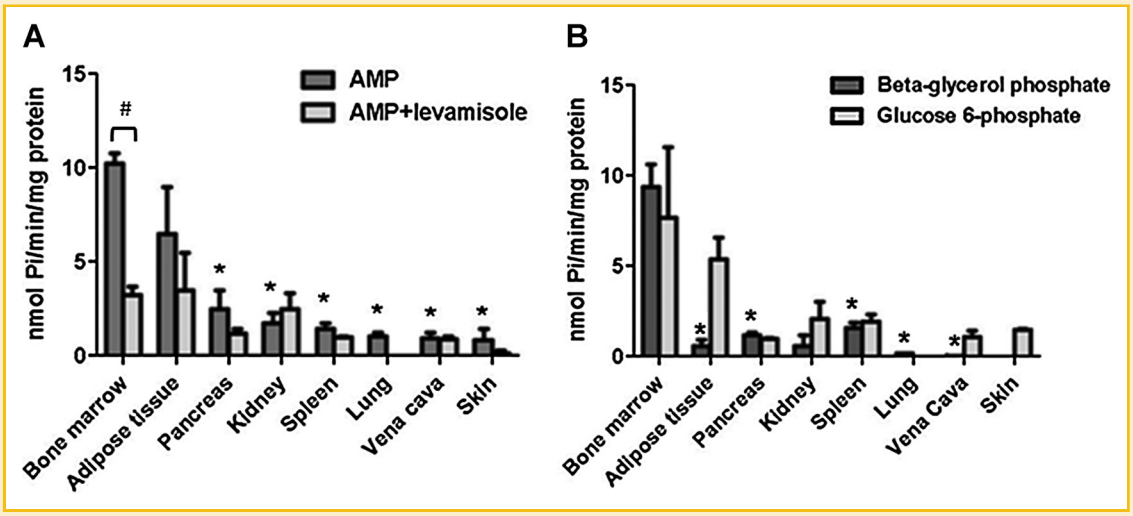

Fig. 4. Extracellular hydrolysis of AMP, glucose-6-phosphate, and β-glycerophosphate by MSCs. MSCs from pancreas, adipose tissue, skin, bone marrow, vena cava, kidney, lung, and spleen were submitted to enzymatic assay having AMP as a substrate in the presence or absence of 1 mM levamisole, alkaline phosphatase inhibitor (A). Cells were also tested by their capacity to hydrolyze β-glycerophosphate or glucose-6-phosphate, alkaline phosphatases substrates (B) as described in the Material and Methods section. Specific activity was expressed as nmol Pi/min/mg protein. Data represent the means of three independent experiments performed in triplicate ± SEM. In some instances, the activity values are too small to be detectable. $P < 0.05$ was considered significant. Data were analyzed statistically by one-way ANOVA followed by Tukey post-test. A: *Different from bone marrow. #Difference between AMP and AMP + levamisole in bone marrow-derived MSC. B: *Different from bone marrow.

These cells have acquired a promising role for cell therapy in many diseases due to their easy access, isolation, and culture. ATP is the most concentrated intracellular metabolite and therefore a fundamental signaling molecule of cell lysis as well as of other pathological and physiological conditions. Therefore, degradation of this nucleotide plays important roles in the control of the effects of ATP and their degradation products.

ATP is able to induce differentiation and proliferation of neuronal precursor cells via P2Y receptors and extracellular nucleotides are considered molecules that contribute to the maintenance of neurogenesis in adult mice [Shukla et al., 2005; Mishra et al., 2006; Jia et al., 2009]. Additionally, ATP-stimulated bone marrow derived hMSCs down regulated genes involved in cell proliferation, whereas those that were involved in cell migration were strongly upregulated. Furthermore, the pretreatment with ATP significantly increased the *homing* capacity of human MSCs in vivo [Ferrari et al., 2011]. These results indicate that the presence of nucleotides in the extracellular space may be required by cells in certain circumstances to keep their undifferentiated state, induce cell differentiation, migration or proliferation. Cancer stem cells (CSCs) seem also to be influenced by purinergic signaling. For example, in glioma CSC, ATP, which can activate P2Y and P2X receptors, reduces the number and size of spheres, increases the number of cells needed to form a sphere, and decreases the number of positive cells to markers of embryonic stem cells, suggesting an important prodifferentiative and antiproliferative role of ATP in these cells [Ledur et al., 2012]. Likewise, P19 embryonic carcinoma cells, used frequently as a model for neuronal and glial differentiation, also can suffer influence of purinergic system, once the treatment with three different antagonists of purinergic P2Y and P2X receptors decreased the differentiation of P19 NSCs to P19 neurons. In the same work, P2X3, P2X4, P2Y1, and P2Y4 receptor expression decreases during the course of differentiation, while gene and protein expression of P2X2, P2X6, P2Y2, and P2Y6 receptors increased, suggesting that activation of these receptors is pro-differentiative for NSCs [Resende et al., 2007].

Little is known about the ectonucleotidases that are responsible for the control of nucleotides levels on cell surface of MSCs. Therefore, in the present study, we tested the ability of MSCs, obtained from different tissues of adult mice, to hydrolyze nucleotides as well as the ectonucleotidases expression profiles presented by these cells. The differences in 10 orders of magnitude of ecto-nucleotidase activities presented by MSCs isolated from different sources indicate that their role in these tissues regarding

TABLE IV. Specific Activities for the Hydrolysis of Alkaline Phosphatase Substrates, β-Glycerophosphate, and Glucose-6-Phosphate

	Specific activities (nmol Pi/min/mg of protein)	
Cellular origin	β-Glycerophosphate	Glucose-6-phosphate
Bone marrow	9.36 ± 1.25	7.66 ± 3.9
Spleen	1.58 ± 0.28	1.89 ± 0.41
Pancreas	1.16 ± 0.16	0.94 ± 0.06
Adipose tissue	0.58 ± 0.33	5.34 ± 2.20
Kidney	0.57 ± 0.57	2.07 ± 0.96
Lung	0.18 ± 0.00	No detected
Vena cava	0.02 ± 0.00	1.07 ± 0.33
Skin	No detected	1.44 ± 0.06

Results are expressed as mean ± standard error of mean (SEM) of at least three experiments for substrate hydrolysis.

TABLE V. Expression of Ectonucleotidases mRNA as Determined by RT-PCR

Genes	Adipose tissue	Vena cava	Kidney	Bone marrow	Lung	Pancreas	Spleen	Skin
NTPDase 1	+	+	+	+	+	+	+	+
NTPDase 2	–	+	+	+	+	+	+	+
NTPDase 3	+	+	+	+	+	+	–	–
NTPDase 5	+	+	+	+	+	+	+	+
NTPDase 6	+	+	+	+	–	+	+	+
E5NT/CD73	+	–	+	+	+	+	–	+
β actin	+	+	+	+	+	+	+	+

+, detected; –, no signal detected.

to the control of extracellular ATP in their microenvironment may vary considerably, adding to other differences found among MSCs isolated from different tissues [da Silva Meirelles et al., 2009]. A high degradation rate of the MSC of a given tissue may reflect the importance of extracellular nucleotides in this tissue, as is the case of the pancreas, where NTPDases 1–3 were shown to be very active. NTPDase1 is localize in all blood vessels and acini, NTPDase2 is present in capillaries of Langerhans islets and in peripheral conjunctive tissue, whereas NTPDase3 can be detected in all Langerhans islet cell types, indicating the importance of the purinergic system in the control of the levels of extracellular nucleotides in this tissue [Lavoie et al., 2010].

It is interesting to observe that the relation among the degradations kinetics of ATP, ADP, and AMP and the expression level of the different *ntpdases* vary considerably. For example, the ATP/ADP ratio varied more than 30-fold, from 10 in MSC from skin to 0.3 in MSC from spleen, whereas the ATP/AMP + levamisole ratio varied from 17 in adipose-derived MSCs to 0.7 in MSC from the spleen (Table VI), therefore, affecting the relative concentration of these nucleotides in the extracellular milieu in these tissues. In astrocytes obtained from different brain regions, the ATP/ADP ratio varied only by a factor of 2 whereas the ATP/AMP ration varied 11-fold when comparing astrocytes from hippocampus and cerebellum, suggesting that these large variations are observed even if cells originate from a different region of a given organ [Wink et al., 2003a]. However, the relation between the expression level of different enzymes and the level of nucleotide degradation indicates that in MSC from some tissues, such as the lung, a high level of expression of several *ntpdases* did not lead to a high level of degradation, suggesting regulation of translation or post-translational regulation [Wink et al., 2000].

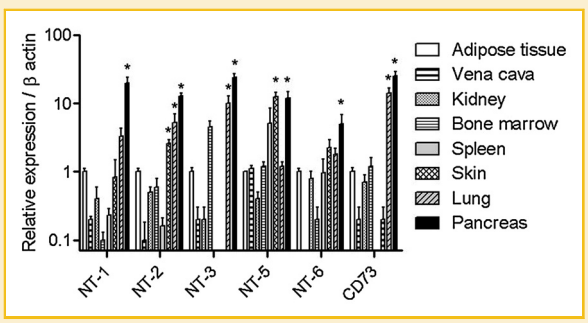

Fig. 5. Ecto-nucleotidases mRNA expression in MSCs from different murine tissues. MSCs from pancreas, adipose tissue, vena cava, kidney, lung, skin, spleen, and bone marrow were analyzed by RT-PCR and quantified by real-time PCR analysis, as described in the Materials and Methods section. NT was used as shorthand for NTPDase. Data represent mean ± SD. Symbols denote significance $P < 0.05$ by one-way ANOVA followed by Tukey post-test. *Different from adipose tissue.

Extracellular nucleotides, mainly ATP, have been increasingly implicated in immunomodulation, mainly in the process of immunogenic cell death (ICD) [Martins et al., 2013]. Inhibition of ecto-ATPases increased the immunogenic effects of chemotherapeutic agents in colorectal carcinoma cells and over-expression of NTPDase1 (CD39) in these cells significantly reduced the immunogenicity, and therefore, the therapeutic effect of mitoxantrone [Michaud et al., 2011]. Since the extracellular metabolism of ATP of one cell type affects the microenvironment, presence of MSCs with high ATPase activity may reduce the therapeutic effects of agents that induce ICD and therefore the nucleotide metabolism of MSCs used in these studies has to be evaluated in order to maximize the potential of these interventions [Nakamizo et al., 2005; Beckermann et al., 2008; Chanda et al., 2009; Bianchi et al., 2012; Vegh et al., 2013].

In conclusion, we described a substantial difference in the extracellular nucleotide degradation rates among MSCs from different murine tissues and showed that this difference is related to differential E-NTPDase and eNT/CD73 expression profile presented by these cells. Our results can contribute to better understanding the biology of MSCs derived from different sources and open perspectives for future investigations about the involvement of purinergic signaling during MSCs differentiation, migration, and proliferation.

TABLE VI. Hydrolysis Rates of Nucleotides by MSC From Different Murine Tissues

	ATP/ADP	ATP/AMP + levamisole	ADP/AMP + levamisole
Pancreas	1.8	6.5	3.5
Adipose tissue	2.4	17.3	7.3
Skin	10.0	4.0	0.4
Vena cava	1.7	5.3	3.2
Kidney	0.6	–	–
Lung	1.3	2.3	1.7
Bone marrow	0.6	0.95	1.57
Spleen	0.3	0.7	2.2

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