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In vitro Pneumovirus and Paramixovirus infection is modulated by the passage of mesenchymal stem cells

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Abstract

The human respiratory syncytial virus (hRSV), the human metapneumovirus (hMPV), and the human parainfluenza virus (hPIV) are the main etiological agents of acute respiratory infections in children and adults. Human mesenchymal stem cells (hMSCs) are also infected by these viruses. These cells differentially express receptors according to the number of passages, thereby, influencing susceptibility and permissiveness to viral infections. The aim of this study was to determine the susceptibility of amniotic hMSC to hRSV, hMPV, and hPIV. We examined the effect of hMSC passages on the viral gene expression by endpoint RT-PCR and the viral production by $TCID_{50}$ and evaluated the effect on the hMSC cytoskeleton by light microscopy. We found that the viral titer increased with respect to the number of hMSC passages. This coincided with the highest gene expression levels documented at the same passages. As for the hMSC morphological changes, we suggest that these changes were associated with actin modifications. Taken together, viral infections of

hMSCs cause altered gene expression and cytoskeleton morphology, with the viral loads ascending as a function of the number of passages.

Keywords: Mesenchymal stem cells; human respiratory viral infection; hRSV; hMPV; hPIV; cytoskeleton; susceptibility; permissiveness.

1. Introduction

Mesenchymal stem cells (MSC) are excellent candidates for cellular therapies (e.g., regenerative medicine and inflammatory disorders, immune-mediated inflammatory diseases) due to their physiological properties, such as their immunoregulatory functions [1,2]. However, MSC therapy can result in enhanced viral replication, particularly when they are highly permissive to infection [3] due to the presence of numerous functional surface receptors [3,4,5] that potentially facilitate viral entry.

MSCs are highly susceptible to viral infections by a variety of DNA viruses, like Herpes simplex virus type 1, Varicella zoster, Hepatitis B, or Parvovirus B19, and RNA viruses, such as human, swine, and avian influenza virus H1N1, human immunodeficiency virus, or Chikungunya virus [6–13]. Among the RNA virus, the human respiratory viruses Pneumovirus and Paramixovirus cause respiratory viral infections that lead to significant morbidity and mortality in patients with hematopoietic stem cell transplants [14].

The human respiratory syncytial virus (hRSV) and the human metapneumovirus (hMPV), members of the *Pneumovirus* family, together with the human parainfluenza virus (hPIV 1–3), member of the *Paramyxovirus* family, are the main etiological agents of acute respiratory infections in both children and adults [16]. Furthermore, they have severe effects on patients with stem cells transplants, including death (between 20% and 40% of the cases) [9,16–19]. Human MSCs (hMSCs) can be infected by hRSV [3,9], which leads to the expression of different cytokine and chemokine profiles [20]. Furthermore, there is some evidence showing the permissiveness of lung-resident MSCs to hRSV infection [21]. Consequently, an antiviral response is triggered producing a variety of immune-modulatory mediators, including interleukins and interferons, that may have a biological impact on the pulmonary microenvironment [21]. However, the consequences of the viral infection on biological events other than immune modulation are poorly understood.

This study aims to examine the changes of hMSCs after Paramixovirus and Pneumovirus infection. First, we determined the susceptibility of amniotic hMSC to viral infection. Then, we examined whether the viral gene expression and viral production were restricted by the number of hMSC passages. Finally, we investigated the effect of the viral infection on the hMSC cytoskeleton by evaluating morphological changes, known as the cytopathic effect (CPE). Briefly, we corroborated the susceptibility and permissiveness of hMSC to hRSV, hMPV, and hPIV. Second, the number of passages played a key role in the production of viral progeny. Finally, we documented CPE associated with cytoskeletal modifications.

2. Materials and Methods

2.1 Cells and viruses

hMSCs were obtained and cultivated as previously described [25,26]. A human placenta was recovered during a C-section in the Hospital Regional de Alta Especialidad, ISSSTE Tultitlán de Mariano Escobedo, México and was transported to the Laboratory of Biology of Cytoskeleton and Virology. The placenta was transported to the laboratory of Biology of Cytoskeleton and Virology in the Facultad de Medicina (UNAM), to collect the amniotic membrane and cultivate the hMSCs. Both processes have been previously described in [25,26]. Human laryngeal epithelial cells (HEp-2; ATCC CCL23) and Vero cells (normal adult African green monkey kidney cells; ATCC CCL81) were used to multiply the viral stocks for the viral titer test They were propagated as described in [27]. The viruses hRSV Long [subgroup A (hRSV-A; ATCC® VR-26™)], hRSV 18537 [subgroup B (hRSV-B; ATCC® VR-1400™)], hPIV2 (ATCC® VR-92™), and hMPV were clinically isolated in our laboratory. The procedures for propagating the viruses and assessing the viral infectivity are described in [27].

2.2 Viral infection kinetics of hMSCs

Monolayers of confluent hMSCs were infected with the viral stocks at different multiplicities of infection (MOI). The MOI were selected for each virus according to our preliminary results of viral infections and are the following: 0.2 for hPIV2, 0.5 for hRSV-A, and 1 for hRSV-B and hMPV. The kinetics of infection were evaluated in hMSC passages P3, P5, P7, and P9 at 18 h, 24 h, 48 h, or 72 h post-infection (PI). Briefly, viral stocks were added to an hMSCs monolayer and allowed to incubate for 2 h at 37 °C. Thereafter, the non-absorbed virus was removed, and fresh Dulbecco's minimum essential medium was added. After each time point,

we observed the cytopathic effect (CPE), defined as the changes in cell morphology (syncytium) by light microscopy (Nikon Eclipse TS 100). The supernatants were collected at 48 h PI and were titrated by $TCID_{50}$ (Tissue Culture Infectious Dose). $TCID_{50}$ was calculated according to the Kärber formula [27].

2.3 Viral gene expression after infection of hMSCs

Total RNA was isolated from hMSCs using Trizol at 18 h PI when cell damage was minimum, and we obtained the required quantity of RNA for the molecular assays. The synthesis of cDNA and the PCR amplification were performed for each gene (gene GA for hRSV-A; gene GB for hRSV-B; gene NH for hPIV-2; and gene N for hMPV) as described in Perezbusta et al. [28]. All PCR products were analyzed on 1% agarose gels and stained with ethidium bromide. The DNA bands were visualized by examining each gel under UV light and were documented on GelDoc Hood II (BioRad).

3. Results

3.1. The number of passages of hMSCs did not restrict the viral infection

hMSCs' susceptibility and permissiveness to viral infections were evaluated as a function of the number of passages. We found that all hMSC passages were permissive to the viral infection. CPE was evident at 24 h PI in the case of hPIV2 (Fig. 1I), even at an MOI of 0.2, and 48 h PI in the case of hRSV-A (Fig. 1F), hRSV-B (Fig. 1G), and hMPV (Fig. 1H). The CPE size was smaller than that of the distinctive syncytium observed in the cell lines Hep-2 (Fig. 1B, C, D) and Vero (Fig. 1K), and the morphological changes were not typical of the syncytium. These changes suggest a modification of the hMSC cytoskeleton after a viral infection compared to a mock infection (Fig. 1A, E, J).

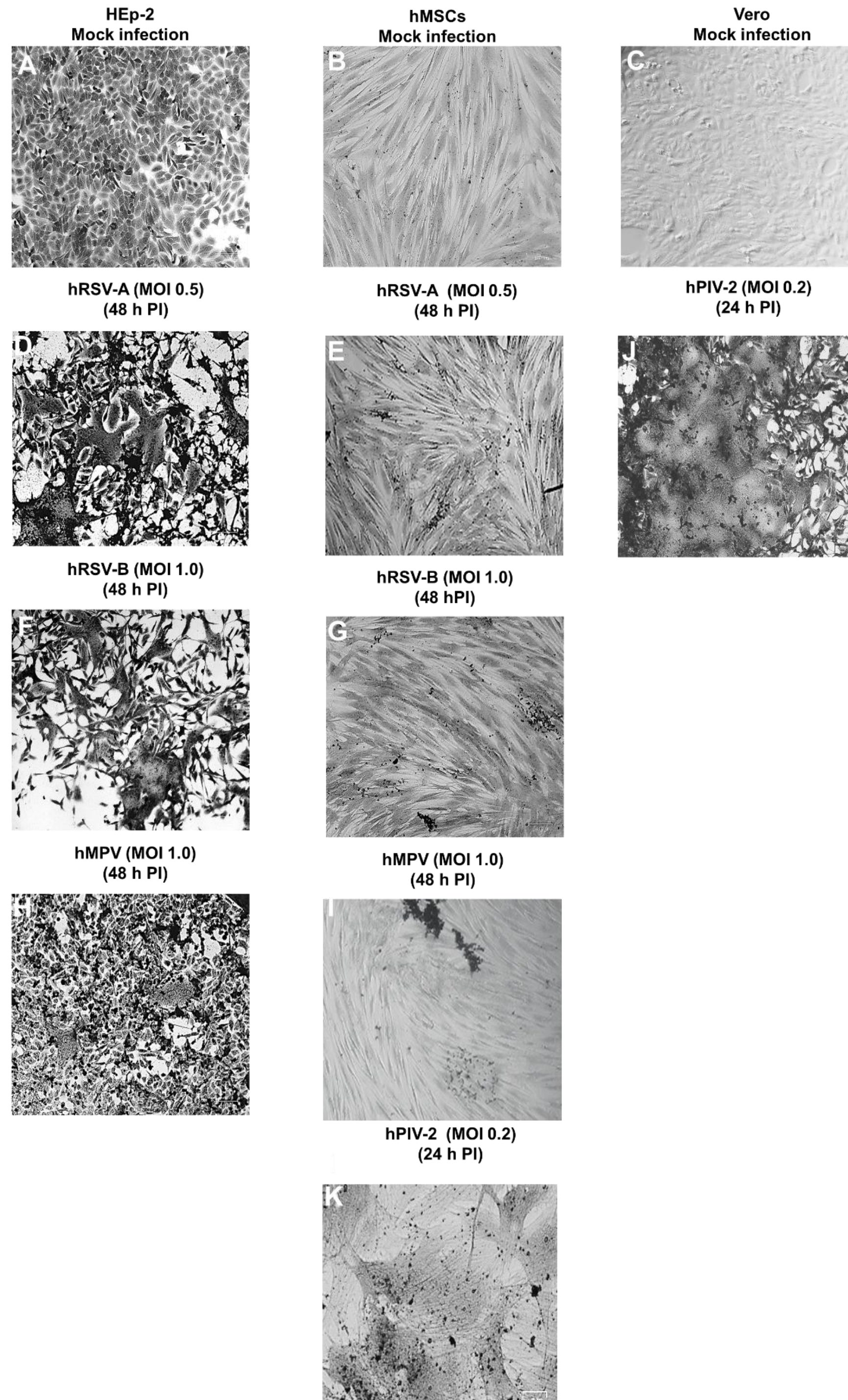

Figure 1. The viral infection causes morphological changes in human mesenchymal stem cells (hMSCs). Representative images of mock-infection (A) Hep-2, (B) MSCs, and (C) Vero. (D) Infection of Hep-2 with hRSV-A caused a distinctive syncytium, (E) hRSV-A infected hMSCs cause morphological changes (CPE), but not a distinctive syncytium, (F) Infection of Hep-2 with hRSV-B cause the distinctive syncytium, (G) hRSV-B cause CPE in infected hMSCs, (H) Distinctive syncytium formation in hMPV infected Hep-2 cells, (I) Infection of hMSCs with hMPV only caused CPE, and hPIV-2 caused in both (J) Vero and (K) MSCs the formation of syncytium.

3.2. hMSC passages and viral progeny

The next question was if the hMSC passages were a restriction factor for the production of viral particles. Based on the results of hMSC morphology, we collected the supernatants at 48h PI and determined the viral titers by $TCID_{50}$. It was shown that the viral titer increased alongside the number of passages. Nonetheless, this increase was only evident between hMSC passages P3 and P7 for all the viruses. Among them, the viral titers follow the order hRSV-A > hPIV-2 > hRSV-B > hMPV (Table 1).

Table 1. Extracellular production of the utilized viruses by the infected hMSCs. The extracellular viruses (supernatants) were collected at MSCs passages P3, P5, P7, and P9 after 48 h of infection. The titer of the viruses increased between passages P3 and P7 independently of the applied virus. However, at P9, we observed a decrease of the viral titer in the cases of hRSV-A, hRSV-B and hPIV-2, these results were associated to changes in the pattern of expression of the molecules that function as viral receptor an/ or changes in the permissiveness related to the passages of the MSCs. This titer reduction non observed in the case oh hMPV infection, probably associated to a major receptor expression and a better permissiveness.

Table 1. Determination of extracellular virus by $TCID_{50}$/ml					
Virus	Titer	hMSCs P3	hMSCs P5	hMSCs P7	hMSCs P9
hRSV-A	$1x10^7$	$1x10^6$	$1x10^8$	$1x10^{11}$	$1x10^5$
hRSV-B	$1x10^5$	$1x10^4$	$1x10^5$	$1x10^9$	$1x10^8$
hPIV-2	$1x10^9$	$1x10^4$	$1x10^3$	$1x10^{10}$	$1x10^5$
hMPV	$1x10^4$	$1x10^5$	$1x10^5$	$1x10^6$	$1x10^7$

3.3. Viral gene expression according to the passages of the infected hMSCs

Finally, we evaluated the viral gene expression by endpoint RT-PCR in the infected hMSCs at different passages (P3, P7, and P9) of the following genes: GA (549 bp) for hRSV-A, GB (109 bp) for hRSV-B, NH (279 pb) for hPIV2, and N (599 bp) for hMPV. Viral expression was positive in the infected hMSC at passages P3, P5, and P7 at 18 h PI (Table 2), confirming the susceptibility and the permissiveness of this cell type. Nevertheless, at passage P9, the product of amplification of GA was not detected (Table 2), even when the samples were positive for CPE and increased viral titers were quantified by $TCID_{50}$. Probably, in the case of passage P9, the assay should be performed between 24 h and 48 h PI to amplify this specific gene (i.e., GA).

Table 2. Viral gene expression in infected MSCs was determined by endpoint RT-PCR. Total RNA was extracted from infected MSCs at passages P3, P5, P7, and P9 at 18 h post-infection. The viral gene expression of hRSV (GB), PIV-2 (NH) and hMPV (N) were detected an all the passages P3 to P7, except hRSV-A (GA), associated to the multiplicity of infection or the hours post infection. bp (base pairs).

Table 2. Viral gene expression in infected hMSCs determined by endpoint RT-PCR

Virus	Viral gene	Endpoint amplification product
hRSV-A	GA	none detected (549 bp)
hRSV-B	GB	Positive (109 bp)
hPIV 2	NH	Positive (279 bp)
hMPV	N	Positive (599 bp)

4. Discussion

In this study, we demonstrated the following: 1) the increase of the viral titer occurs as a function of the number of hMSC passages, 2) the viral gene expression depends on the viral titers and the hMSC passages, and 3) the infected hMSCs presented CPE corresponding to the duration of the infection. Our results reveal the susceptibility of hMSCs to different human respiratory viruses that cause acute respiratory viral infections, as shown by CPE (Figure 1). These viruses specifically infect the ciliated epithelial cells of the respiratory mucosa of the nose and throat, causing upper and lower respiratory tract diseases [29–32]. In this context, our results are relevant as they suggest that these respiratory viruses infect progenitor cells, like airway basal epithelium cells [29]. When this event occurs, the viral infection of hMSCs (considered progenitor cells) affects diverse biological structures and functions, like the conducting airways or the repairment and differentiation of the respiratory epithelium, which preserves the mechanical barrier against respiratory pathogens [29].

Our results agree with a recent report [29] that describes an *in vivo* model of hRSV infection of the airway's basal cells that function like progenitor cells, where the consequences of the viral infection had a significant and long-term impact on the biology of the airway epithelium. The change of the epithelial phenotype may contribute to the excessive mucus production and

the occlusion of the small airways that occur following hRSV infection [29]. This event possibly occurs in the case of other respiratory viruses, too.

In our *in vitro* model, all respiratory viruses caused a productive infection, as demonstrated by the quantification of the extracellular virus in the supernatants obtained from the infected hMSCs (Table 1). In our experiments, the infection depended on the hMSC passage at which the cells were infected and the respiratory virus used. The differences in the productive infection suggest that the susceptibility and the permissiveness of hMSCs are related to differential surface receptor expression occurring with respect to the number of passages.

Additionally, the viral gene expression for all studied genes was detected by endpoint RT-PCR, except for hRSV-A's GA gene. Since the extracellular virus was detected under the same conditions, meaning that there was a productive viral infection, this result could be due to the applied MOI or the duration of infection. If these conditions were manipulated, they would probably permit the detection of the GA gene.

Our experiments showed that the viral infection possibly contributes to changes in the epithelial phenotype, a modification that is a risk factor for severe respiratory viral infections (bronchitis or bronchiolitis), asthma, and chronic obstructive pulmonary disease [29,33,34]. Finally, it is important to mention the relevance of this study in hMSC therapy of virus-induced diseases, especially in the case of prolonged inflammation or immunomodulation. In this context, hMSC therapy may result in enhanced viral replication in certain viral infections, particularly when MSCs are highly permissive to infection.

In conclusion, our results suggest that the studied human respiratory viruses can be considered a risk factor because of hMSC tropism. These cells, like the progenitor cells of the respiratory airways, could present affected biological functions leading to the imminent possibility of infection in hMSC therapy.

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Institutional Review Board Statement The study was conducted in accordance with the biosafety regulations of the World Medical Association's Declaration of Helsinki regarding the ethical conduct of research involving humans and was approved by the Research Ethics Board of Facultad de Medicina, UNAM (project number 101-2012).

Informed Consent Statement Informed consent was obtained from all subjects involved in the study. The informed consent letters were approved by the Committee of Ethics and Research of the División de Investigación de la Facultad de Medicina de la UNAM (FMED/CI/SPLR/004/2016).

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