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Original Article

Kinetics of Nitric Oxide Production and MTT Reduction by HSV-1 Infected Macrophages

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Abstract

Background: Macrophages have important role in defense against Herpes Simplex Virus type-1 (HSV-1). The present study was performed to determine the viability and nitric oxide (NO) production by HSV-1 infected mouse peritoneal macrophages (HIM).

Method: The viability of macrophages was evaluated using MTT reduction assay and the production of nitrite using Griess method.

Results: The ability of infected macrophages to reduce Tetrazolium (MTT) was diminished at virus to cell ratios of multiplicity of infection (MOI) of one, three and 10; but not at 0.01and 0.1. Induction and inhibition of NO production by HIM were MOI dependent. The basal NO production by these cells was inhibited at MOI of three and ten. In contrast virus to cell ratios of 0.01 and 0.1 induced low but significant enhancement in NO production. The inability of HIM to reduce MTT at MOI of three was significant after 12-hrs and inhibition of NO production was initiated between 12-20 hours after infection.

Conclusion: High doses of HSV-1 seem to decrease the normal activity of macrophages by inhibiting the production of nitric oxide.

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Keywords ● HSV–1 ● macrophage ● nitric oxide ● MTT assay

Introduction

Macrophages are important components of defense against herpes simplex virus type-one (HSV-1).[1,2] They produce different cytokines and metabolites in response to virus infections.[3,4] Among different products of macrophages in response to HSV-1, various indirect studies, including inhibition of nitric oxide synthase (NOS),[5,6] or NOS gene knocking out mice,[7] have shown that nitric oxide (NO) is an important mediator. Despite this, it is not clear yet whether the virus itself is able to directly induce NO production by macrophages and to what extent this issue is related to macrophage viability.

MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) reduction assay is widely used to evaluate cell viability and proliferation. MTT reduction is interpreted to be indicative of cellular metabolic activity.[8] Herein, the macrophage susceptibility to HSV-1 and kinetics of NO production by HSV-1 infected macrophages are studied using MTT assay as one of

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the most sensitive tests in this regard.[9]

Materials and Methods

Virus

Vero cell line (NCBI C101) was grown in RPMI-1640 medium supplemented with 10% fetal cuff serum (FCS; Seromed, Germany) using 96-well microplates (Falcon, USA) and infected with a well-known strain of HSV-1 (KOS strain). Virus titer was determined by plaque forming assay on Vero cells.[10] Briefly, $3X10^5$ Vero cells were cultured on a 24 well microplate to form a monolayer which were then inoculated with dilutions of virus ranging from 10^{-2} to10^{-9} prepared in duplicate in culture medium. After one hr adsorption, the remaining media were removed and replaced with medium containing 0.5% agarose and 5% FCS. After 72-hrs the plaques were counted following fixation with formaldehyde (10% in PBS), and staining with crystal violet.[10] The aliquots of virus stock were prepared and stored at -70°C until use.

Macrophage culture and infection

Peritoneal cells obtained from 8-10 weeks-old male Balb/c mice were washed twice in cold PBS phosphate buffered saline (PBS), resuspended in cell culture medium (RPMI) supplemented with 10% FCS and added to 96-well microplates ($4X10^5$ cells/well) followed by incubation at 37°C for two hrs under 5% CO2. The non-adhering cells were then removed by washing with PBS, pre-warmed to 37°C.[11] About half of the cells (54±4%) were removed as determined by counting the washed cells from six wells. More than 95% of adherent cells were macrophages as shown by Giemsa staining. The adherent cells were infected with the virus at several multiplicity of infection (MOI) or mock infected. The supernatant of uninfected Vero cells in similar conditions were used for mock infection. Viral adsorption was allowed to proceed for one hr followed by addition of growth medium, supplemented with 10% FCS, to a final volume of 250µl and incubation of microplates under 5% CO2 at 37°C. All media and solutions used were endotoxin free.

Nitrite assay

The production of NO by macrophages was assayed by measuring nitrite in the supernatant of cultured macrophages. Briefly, 100µl of supernatant from each well was transferred to a 96-well flat-bottom microtiter plate and 50 µl of 1% solution of sulfanilamide (Fluka, UK) and 50 µl of 0.1% N-(1-Naphtyl)-ethylen-diamin-dihydrochloride (NEDA) solution (Merck, Germany), both in 5% phosphoric acid (Merck, Germany), all were added to samples and to $NaNO_2$ standards (0-100 nmol) prepared in RPMI medium. After 10 min the absorbances were read at 540 nm.[12]

MTT assay

MTT (Merck, Germany) was dissolved in PBS (5mg/ml), filtered and stored at –20°C until use. MTT solution was added to each well at one tenth of its volume (25µl) and the supernatants were gently removed after four hrs. The formazan crystals were dissolved in acidic isopropanol (0.04 M HCl in isopropanol) and their absorbance was recorded at 540 nm.[13]

Statistical analyses

Data are presented as mean±SEM. Macrophage viability and activity (the results of MTT reduction assay) of mock infected control and HSV-1 infected groups were compared with each other using one-way analysis of variance (ANOVA) followed by Tukey tests. Nitric oxide production of mock infected control and various HSV-1 infected groups were compared with each other as well and $P<0.05$ was considered as the statistically significant between groups.

Results

NO production could be either induced or inhibited in HSV-1 infected macrophages, depending on the MOI or virus to cell ratio. As shown in Fig. 1, the basal level of NO production of infected macrophages, in comparison with mock-infected controls, were diminished significantly ($p<0.001$) at high MOI (3 and 10), whereas, it was elevated to 1.6 and 2.1 times at low MOI (0.01 and 0.1) respectively ($p<0.05$).

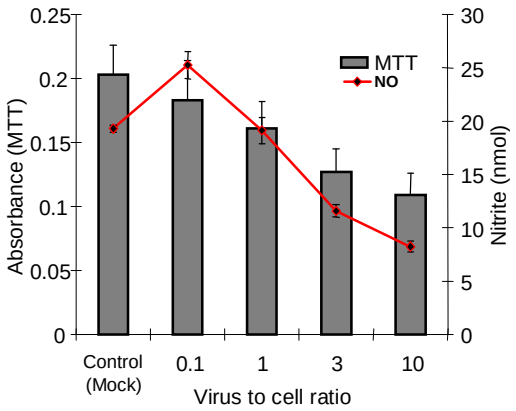

Fig 1: The values (mean±SEM) of MTT reduction and NO production by mouse peritoneal macrophages infected with HSV-1 group at various MOI and control group. The number of macrophages was $2x10^5$/well. MTT reduction ability of infected macrophages was diminished at MOI of 1, 3 and 10. NO production by infected macrophages was also inhibited at MOI of 3 and 10 ($p<0.05$). Infection of macrophages at MOI of 0.1 and 0.01 did not alter their ability to reduce MTT but induced an augmentation of NO production ($p<0.05$).

Macrophage activity evaluated by MTT assay has been diminished significantly (P<0.05) at MOI of one, three and 10 to 77% 59% and 40% of their control (mock infected) after 24 hrs respectively. However, low doses of the virus (MOI of 0.01, and 0.1) did not significantly change the activity macrophages as compared with their control (mock infected).

Kinetics of inhibitory effects of HSV-1 on NO production and MTT reduction in infected macrophages

Infection with HSV-1 (MOI=3) inhibited basal NO production by freshly cultured mouse peritoneal macrophages (2X10^5/well). As shown in Fig 2A, NO production decreased at 12 to 20-hrs of infection in macrophages infected with HSV-1, but the difference from control (mock infected) was only significant at 20-hrs of infection.

The ability of infected macrophages to reduce MTT was impaired with time (Fig 2B) and the significant differences from control was observed at 12-hrs of infection (P<0.05). MTT reduction by infected macrophages was decreased to 65% at 32-hrs of infection. On the other hand, the viability and the activity of uninfected macrophages were not altered significantly at this time.

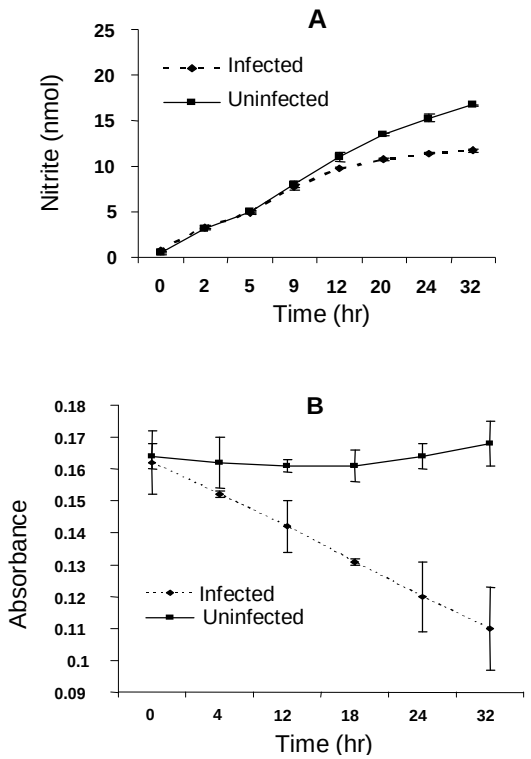

Fig 2: The Values (mean±SEM) of NO production (A) and MTT reduction (B) by HSV-1 and mock infected (control) mouse peritoneal macrophages. The number of macrophages was 2x10^5/well and MOI was 3. The declined NO production by HSV-1 infected macrophages at about 12-hrs after infection was significantly different from that of control at 20-hrs. A significant decline in MTT reduction by infected macrophages was also observed 12-hrs after infection (p<0.05).

Discussion

The interplay between macrophages with infectious agents forms a critical part of immune response, because macrophages are usually the first line of defense against infectious microorganisms and perform both effector and accessory functions.[14] Macrophages have important role in defense against, and are partly permissive to HSV-1 infection.[15-18]

We have studied the effect of infection with various loads of the virus on viability and activity of macrophages using MTT assay.[9] It should be emphasized that MTT assay is suitably applied to purposes such as screening antiviral compounds against HSV-1,[19,20] or HIV induced cytopathogenicity in macrophages,[21] and due to its simplicity and reproducibility, is preferable to other tests.

As shown in Fig 1 macrophages were able to resist low MOI of the virus without any failure in their MTT reducing ability, whereas, they gradually lost this capability at higher doses of the virus. In regard to the importance of mitochondria in MTT reduction,[22] the loss of this ability could be partly attributed to HSV-1 induced changes in mitochondrial activity, although, other mechanisms may be involved too. As reported by Murata and colleagues in HSV-1 (KOS strain) infected Vero and Hep-2 cell lines, mitochondrial activity diminished about 12 hrs after infection.[23] Disruption of mitochondrial membrane was also reported in herpes-infected Vero cells.[24]

Macrophages are capable of destroying microorganisms to produce various metabolites, of which NO is one of the most important antiviral compounds.[14,25] Although, induction of NO production in macrophages by the virus itself has not yet been directly demonstrated, it was previously reported in presence of HSV-1 and other stimulators like phorbol myristate acetate (PMA).[26] In our study, induction of NO production was demonstrated in freshly isolated mouse peritoneal macrophages by the virus itself without adding any stimulator. However, the induction effect of the virus on NO production of macrophages was moderate and could be observed only at low MOI. This finding is in agreement with the conclusion that the production of NO is down regulated by HSV-1.[27] Others also believe that the inhibition of NO production by the virus, that occurs at high MOI, may be attributed to the inhibition of protein synthesis by some viral glycoproteins,[28-30] or HSV-induced disturbance in mitochondrial activity.[23,24] Therefore, more studies are needed to elucidate the mechanisms of NO stimulation or inhibition by HSV-1 and verify parameters which influence these interactions in regulating

the immune response system against HSV-1.

Conclusion

Although, macrophages are the important line of defense against HSV-1, they are vulnerable to high viral loads which inhibit their nitric oxide production and cause cell mortality.

References

1 Kodukula P, Liu T, Rooijen NV, et al. Macrophage control of herpes simplex virus type 1 replication in the peripheral nervous system. *J Immunol* 1999; 162: 2895-905.

2 Cheng H, Tumpey TM, Staats HF, et al. Role of macrophages in restricting herpes simplex virus type 1 growth after ocular infection. *Invest Ophthalmol Vis Sci* 2000; 41: 1402-9.

3 Lokensgard JR, Hu S, Sheng W, et al. Robust expression of TNF-alpha, IL-1-beta RANTES and IP-10 by human microglial cells during nonproductive infection with herpes simplex virus. *J Neurovirol* 2001; 7: 208-19.

4 Paludan SR, Mogensen SC. Virus-cell interactions regulating induction of tumor necrosis factor alpha production in macrphages infected with herpes simplex virus. *J Virol* 2001; 75: 10170-8.

5 Benencia F, Courreges MC, Gamba G, et al. Effect of aminoguanine, a nitric oxide synthase inhibitor, on ocular infection with herpes simplex virus in BALB/c mice. *Inv Ophthalmol Vis Sci* 2001; 42: 1277-84.

6 Croen KD. Evidence for an antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. *J Clin Invest* 1993; 91: 2446-52.

7 McLean A, Wei XQ, Hung F, et al. Mice lacking inducible nitric oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell responses. *J Gen Virol* 1998; 79: 825-30.

8 Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. *J Immunol Methods* 1993; 157: 203-7.

9 Karupiah G, Harris N. Inhibition of viral replication by nitric oxide and its reversal by ferrous sulfate and tricarboxylic acid cycle metabolites. *J Exp Med* 1995; 181: 2171-9.

10 Morgan DM. Tetrazolium (MTT) assay for cellular viability and activity. *Methods Mol Biol* 1998; 79: 179-83.

11 Kinchington D, Kngro H, Jeffires DJ. Design and testing of antiviral compounds. In: Dsselberger U. (Ed), Medical virology: a practical approach, IRL Press; New York; 1995. p. 147-72.

12 Fernandez-Botran R, Vetvicka V. Methods in cellular immunology, CRC Press, Boca Raton; USA; 2001. p. 1-35.

13 Doyle A, Stein M, Keshav, Gordon S. Assays for macrophage activation by cytokines. In "Cytokines: A practical approach" 2ed by Blackwill FR, IRL Press; Oxford; 1995.

14 Oxford JS, Kelly LS, Davies S, Lambkin R. Antiviral testing. In "Virus culture: A practical approach." Edited by Cann AJ, Oxford University Press; Oxford; 1999.

15 Gordon S. Macrophages and the immune response. In "Fundamental immunology" by Paul WE, Lippincott Raven Pub; Philadelphia; 1999. p. 533-45.

16 Carr DJ, Harle P, Gebhardt BM. The immune response to ocular herpes simplex virus type 1 infection. *Exp Biol Med* 2001; 226: 353-66.

17 Berra A, Rodriguez A, Heiligenhaus A, et al. The role of macrophage in pathogenesis of HSV-1 induced chorioretinitis in Balb/c mice. *Invest opphthalmol Vis Sci* 1994; 35: 2990-8.

18 Lopez C, Dudas G. Replication of Herpes Simplex Virus Type 1 in Macrophages from Resistant and Susceptible Mice. *Infect Immun* 1979; 23: 432-7.

19 Plaeger-Marshal S, Wilson LA, Smith JW. Permissiveness of Rabbit Monocytes and Macrophages for Herpes Simplex Virus Type 1. *Infect Immun* 1982; 35: 151-6.

20 Oxford JS, Kelly LS, Davies S, Lambkin R. "Antiviral testing" in "Virus culture: A practical approach" Edited by Cann AJ; Oxford Univ. Press; 1999.

21 Sudo K, Konno K, Yokota T, Shigeta S. A sensitive assay system screening antiviral compounds against herpes simplex virus type 1 and type 2. *J Virol Methods* 1994; 149: 169-78.

22 Bergamini A, Perno CF, Capozzi M, et al. A tetrazolium-based colorimetric assay for quantification of HIV-1-induced cytopathogenicity in monocyte - macrophages exposed to macrophage-colony-stimulating factor. *J Virol Methods* 1992; 40: 275-86.

23 Liu Y, Petrson DA, Kimura H, Schubert D. Mechaism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoilium bromide (MTT) reduction. *J Neuroche* 1997; 69: 581-93.

24 Murata T, Gosshima F, Daikoku T, et al. Mitochondrial distribution and function in herpes simplex virus-infected cells. *J Gen Virol* 2000; 81: 401-6.

25 Tsurumi T, Lehman IR. Release of RNA polynerase rom Vero cell mitochondria after herpes simplex virus type 1 infection. *J Virol* 1990; 64: 450-2.

26 Bodgan C, Rollinghoff M, Diefenbach A. Reactive oxygen and nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol* 2000; 12: 64-76.
27 Lopez-Guerrero JA, Alonso MA. Nitric oxide production induced by herpes simplex virus type 1 does not alter the course of the infection in human monocytic cells. *J Gen Virol* 1997; 78: 1977-80.
28 Thakur A, Athmanathan S, Willox M. The differential regulation of nitric oxide by Herpes simplex virus-1 and -2 in a corneal epithelial cell line. *Clin Exp Ophthalmol* 2000; 28: 188-90.
29 Hobbs WE, DeLuca NA. Perturbation of cell cycle progression and cellular gene expression as a function of herpes simplex virus ICP0. *J Virol* 1999; 73: 8245-55.
30 Parkinson J, Everett RD. Alphaherpesvirus proteins related to herpes simplex virus type 1 ICP0 induce the formation of colocalizing, conjugated ubiquitin. *J Virol* 2001; 75: 5357-62.