Transpl Int (2002) 15: 610–614
DOI 10.1007/s00147-002-0468-z

ORIGINAL ARTICLE

Timi J. Martelius
Henrik Wolff
Cathrien A. Bruggeman
Krister A. Höckerstedt
Irmeli T. Lautenschlager

Induction of cyclo-oxygenase-2 by acute liver allograft rejection and cytomegalovirus infection in the rat

Received: 8 October 2001
Revised 17 June 2002
Accepted: 8 July 2002
Published online: 19 October 2002
© Springer-Verlag 2002

T.J. Martelius (✉) · K.A. Höckerstedt · I.T. Lautenschlager
Department of Surgery,
Transplant Unit Research Laboratory,
Helsinki University Hospital,
Kasarmikatu 11–13,
00130 Helsinki, Finland
E-mail: tmarteli@helsinki.fi
Tel.: +358-9-47188484
Fax: +358-9-47188348

T.J. Martelius · I.T. Lautenschlager
Department of Virology,
University of Helsinki,
Helsinki University Hospital,
Helsinki, Finland

H. Wolff
Department of Pathology,
University of Helsinki,
Helsinki University Hospital,
Helsinki, Finland

C.A. Bruggeman
Department of Medical Microbiology,
University Hospital of Maastricht,
Maastricht, The Netherlands

Abstract Cytomegalovirus (CMV) infection has been shown to increase inflammation in rat liver allografts. In-vitro CMV has been shown to transactivate cyclo-oxygenase-2 (COX-2), while COX-2 plays a role in the CMV replication cycle. Our aim was to investigate the expression of COX-2 in liver allograft rejection and concomitant CMV infection. Expression of COX-2 was studied immunohistologically in rat liver allografts with or without rat CMV infection, in isografts, and in normal rat liver. There were small amounts of COX-2-positive mononuclear inflammatory cells in the normal liver and isografts. Acute rejection increased the amount of COX-2-expressing cells in the portal areas only, whereas concomitant CMV infection did this also in the sinusoid area. COX-2 may play a role in CMV infection in vivo as well. The possible role of COX-2 in the association between CMV infection and allograft rejection warrants further study.

Keywords Cyclo-oxygenase-2 · Liver rejection · Cytomegalovirus

Introduction

Cytomegalovirus (CMV) infection has been linked to acute and chronic liver allograft rejection [2, 5, 11]. We have previously shown that rat CMV increases portal inflammation and bile duct destruction in rat liver allografts with concomitant rejection [13]. Many of the pro-inflammatory effects of CMV have been shown to be mediated by NF-κB activation [8]. In-vitro CMV infection rapidly induces intracellular oxidative stress and, consequently, NF-κB activation [20]. Cyclo-oxygenase-2 (COX-2) has been demonstrated to be involved in this activation cascade in vitro [21]. On the other hand, CMV immediate early 1 (IE1) and 2 (IE2) proteins were shown to transactivate COX-2 promoter, probably also via NF-κB [21]. COX-2 is an inducible immediate early gene and the key enzyme in prostanoid synthesis in the inflammatory response [14]. The study of prostaglandins in the context of liver transplantation has mainly concentrated on ischemia reperfusion injury

[7, 18]. Both immunomodulatory and rejection-promoting effects of prostaglandins have been observed in allografts [15, 19]. In rat cardiac allograft rejection, COX-2 has been shown to be up-regulated [23]. Given these observations, COX-2 could play a role in the pro-inflammatory effects of CMV that we observe in our model of rat liver allograft rejection. COX-2 tissue expression in liver allograft rejection has not been studied previously. The same is true of the effect of CMV on COX-2 expression in vivo. Therefore, we aimed to study the effect of CMV on COX-2 expression in vivo in our model of liver allograft rejection in the rat.

Materials and methods

Rats

A donor–recipient combination of PVG ($RT1^c$) to BN ($RT1^n$) with a previously observed mean survival time of 37 days was used for liver grafting [10]. BN-to-BN syngeneic transplantations were performed for syngeneic controls. The rats were fed regular rat chow and tap water ad libitum. The animals were treated in accordance with the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH). The study was approved by the committee for experimental research at the Helsinki University Central Hospital and the regional authorities.

Transplantation and the rejection model

We performed liver transplantation under ether anesthesia using the technique introduced by Kamada et al. [10], supplemented with reconstruction of the hepatic artery. Cold (4 °C) heparinized 0.9% saline was used for perfusion and preservation of the graft. On day 1 after transplantation the allografted animals in the infection group were infected with rat CMV (see below). No immunosuppressive drugs were given to any of the animals. We have previously demonstrated that, in this strain combination, liver allotransplantation results in prolonged acute rejection with an intense peak of lymphoid activation at 1 week after transplantation, leading to later macrophage-dominated response and advanced tissue damage at a mean survival time of 37 days [12]. Grafts were harvested at 1 week ($n = 5$ in the CMV group, $n = 3$ in the uninfected group) and at 4 weeks ($n = 6$ per group) so that we could observe both the peak of rejection and the more prolonged phase. In addition, four syngeneic liver grafts were performed.

Rat CMV infection

The rats in the infection group were infected by inoculation with 10^5 PFU of rat CMV (Maastricht strain) intraperitoneally 1 day after liver transplantation. The procedure for culturing and inoculating rat CMV as well as the characteristics of rat CMV and rat CMV infection have been described in detail previously [4]. Quantification of the rat virus was done by plaque assay, as described previously by Bruggeman et al. [3]. The infectious virus was stored at −70 °C.

Demonstration of CMV infection

We demonstrated the presence of rat CMV infection in the graft by culturing the virus from material obtained from the graft by fine-needle biopsies. The fine-needle sample was aspirated from the graft into RPMI 1640 culture medium containing albumin. The rat was anesthetized with fentanyl-fluanisone while the fine-needle specimen was obtained. The virus was cultured in rat embryo fibroblasts (REFs) under standard virus culture conditions [4].

Immunohistochemistry

The grafts were harvested under anesthesia, and a portion was fixed in buffered formaldehyde solution for 24 h, while another portion was immediately embedded in OCT compound (Tissue-Tek, Sakura Finetek Europe, The Netherlands), snap-frozen in liquid nitrogen, and stored at −70 °C until use. Formalin-fixed tissue samples were embedded in paraffin, and sections (4–5 μm) were cut. The paraffin was removed and the slides microwaved for 4×5 min in 0.01 M Nacitrate buffer (pH 6.0). The slides were first immersed in 1.6% hydrogen peroxide in methanol for 30 min and then in blocking solution (0.01 M TRIS, 0.1 M $MgCl_2$, 0.5% Tween 20, 1% BSA, and 5% normal goat serum) for 1 h to block endogenous peroxidase activity and unspecific binding sites, respectively. Immunostaining was performed with rabbit polyclonal IgG against mouse COX-2 peptide (Cayman Chemical Company, Ann Arbor, Mich., USA) in a dilution of 1:600 in the blocking solution at 4 °C overnight. The sections were then treated with biotinylated secondary antibody (Vectastain ABC Kit; Vector Laboratories, Burlingame, Calif., USA), and antibody binding sites were finally visualized by avidin-biotin peroxidase complex solution (ABComplex; Vectastain; Vector Laboratories) and 3,3′-diaminobenzidine. As a control, nonspecific rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at a concentration corresponding to that of the COX-2 antibody was used to stain all sections, using an identical protocol. In addition, to show the specificity of the staining, we performed a blocking experiment with COX-2 peptide. Incubation with the primary antibody was done in the presence of 20 μg/ml COX-2 blocking peptide (Cayman Chemical Company). The rest of the staining procedure was performed as above.

The number of positive cells per high-power field (magnification ×400) was counted. For each slide, 20 high-power fields were assessed from both portal and non-portal areas, respectively, and the average was calculated. The cells contained inside the limiting plate of a portal field, or, when demarcation was disrupted by the inflammatory infiltrate, the inflammatory cells in the continuous portal infiltrate, were counted as portal cells. The positive cells found outside the portal fields, in the sinusoidal area, or between the hepatocytes were counted as non-portal/sinusoidal cells. We also counted the total number of mononuclear inflammatory cells per high-power field for portal and sinusoidal areas, respectively, to calculate the percentage of positive cells. Frozen liver sections were also stained with mouse monoclonal antibody ED2 (Serotec, UK), recognizing cells of monocyte/macrophage origin, including Kupffer cells.

Statistics

The results are expressed as mean ± SD, and for assessment of difference between the groups the Mann-Whitney U-test was used. P values of below 0.05 were considered significant.

Results

In this rat model of liver transplantation, there are intense signs of acute rejection at 1 week, with portal inflammation and endotheliitis in the graft. In the later phase, at 4 weeks, there is still portal inflammation, but clearly less than at the peak, and the histological pattern is that of necrosis, fibrosis, and bile duct proliferation in response to injury [12]. CMV infection significantly

increased portal inflammation and bile duct damage in the late phase [13].

Some baseline expression of COX-2 in normal rat liver (data not shown) and syngeneic liver transplants (1.1 ± 0.3 cells/high-power field, 1.2% of mononuclear cells in the sinusoidal area; 1.3 ± 0.3 cells/high-power field, 4.1% of mononuclear cells in the portal areas) was seen. At the peak of inflammation associated with rejection at 1 week after transplantation, the absolute number of COX-2-positive cells in the portal areas increased significantly in both rejection groups (2.3 ± 0.6 in the uninfected and 2.3 ± 0.7 in the CMV group), while in the non-portal area the increase was seen only in the CMV group (2.8 ± 1.2) (Fig. 1). The percentage of COX-2-positive cells in the portal area was actually significantly lower (0.8% and 0.5% in the uninfected and CMV group, respectively) than in the syngeneic group, due to the massive tenfold increase in total mononuclear cells. In the sinusoidal area, the percentage was comparable with the syngeneic group (1.0% and 0.9% in the uninfected and CMV group, respectively). Here, the total mononuclear count increased twofold in the uninfected group and threefold in the CMV group.

At 4 weeks after transplantation, the absolute number of COX-2-positive cells in the portal areas had increased further in both of the rejection groups (4.0 ± 2.4 in the uninfected group and 3.8 ± 1.9 in the CMV group). Again, the number of COX-2-positive cells in the non-portal area was significantly higher in the CMV group than in rejection alone (1.7 ± 1.2 in the uninfected group and 3.7 ± 1.7 in the CMV group, $P < 0.05$) (Fig. 1). At this stage, the percentages of COX-2-positive cells in the portal field was slightly higher than at the peak of inflammation in both rejection groups (1.6% and 1.8% in the uninfected and CMV group, respectively), but still lower than in the syngeneic group. However, in the sinusoidal area, the relative number of COX-2-positive cells was significantly higher in the CMV-infected group than in the uninfected group (2.7% vs 0.9%, $P < 0.05$).

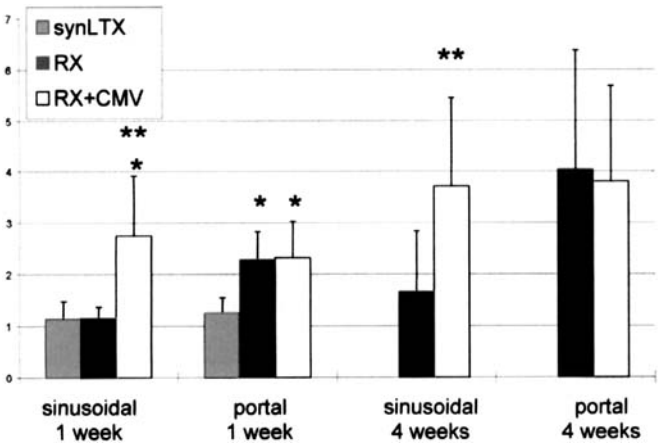

Fig. 1 Number of COX-2-positive cells/high-power field in rat livers (mean ± SD). For each sample, 20 fields were counted and the average was calculated (*synLTX* syngeneic liver transplants, *RX* allografts with rejection, *RX+CMV* concomitant CMV infection and rejection). $*P < 0.05$ (Mann-Whitney U-test, significant difference from syngeneic group). $**P < 0.05$ (Mann-Whitney U-test, significant difference from rejection group)

The localization and morphology of COX-2-expressing cells were similar in all groups, only the amount varied. In the portal fields, the positive cells were mononuclear inflammatory cells in the portal inflammatory infiltrate. Outside the portal fields, the positive cells were found in the sinusoids and between the hepatocytes. These cells were small mononuclear cells with moderate amounts of cytoplasm, and due to their localization, they were most probably Kupffer cells (Fig. 2).

The morphology and distribution pattern of the COX-2-positive cells were compared with cells staining positive with macrophage/Kupffer cell marker ED2. Unfortunately, as COX-2 staining did not work in frozen sections, nor ED2 in paraffin sections, the comparison had to be made between slides stained with different techniques. However, although the morphology of the cells was less well preserved in the frozen sections, we found that the morphology and distribution were quite similar between cells staining for COX-2 and those staining for ED2 (Fig. 2). ED2-positive cells were abundant, and only a fraction of all macrophages/Kupffer cells was positive for COX-2.

COX-2 staining was also seen in vascular smooth muscle cells and at the luminal surface of bile duct cells. However, in contrast to that in the macrophages/Kupffer cells, this staining did not disappear when the specific COX-2 binding was blocked with COX-2 peptide, and was therefore considered nonspecific.

Discussion

This study demonstrates that acute rejection increased the number of COX-2-expressing cells in rat liver allografts. Concomitant CMV infection led to further increase in COX-2 expression. We have previously shown that CMV increases inflammation and graft damage in this model of acute liver allograft rejection [13]. The pattern of COX-2 induction was different in the CMV-infected and uninfected groups undergoing rejection, although in both groups there was vigorous alloresponse. The rejection process seemed mainly to induce COX-2 in the mononuclear cells of the portal inflammatory infiltrate. This is quite logical considering that, in rejection, the inflammatory response concentrates at the portal fields. In the group with concomitant rejection and rat CMV infection, the portal COX-2 expression was similarly increased, but in addition, in contrast to

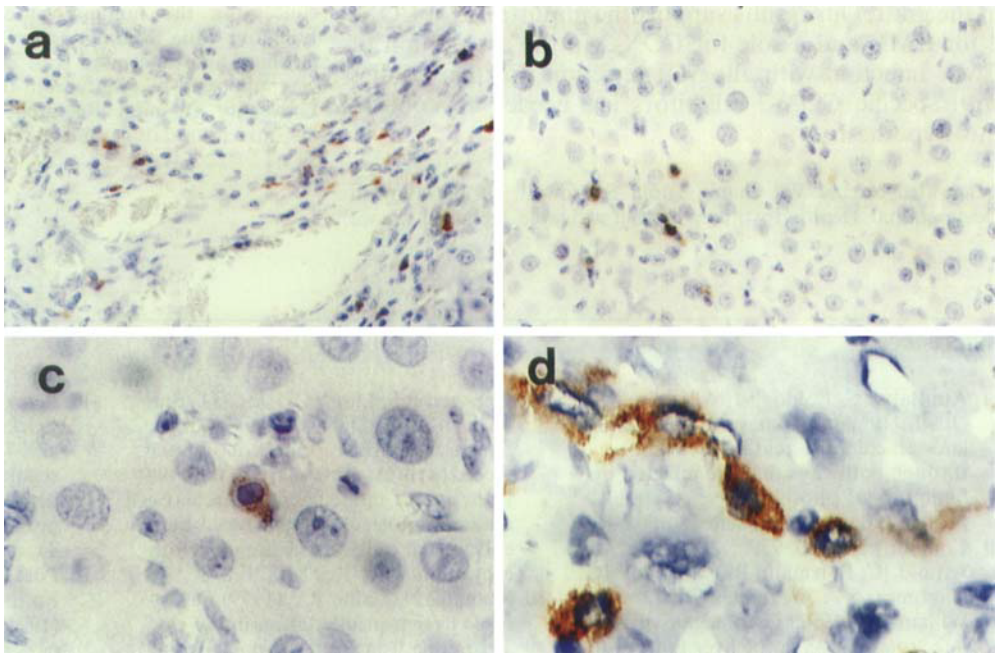

Fig. 2 **a** COX-2-positive cells in the portal inflammatory infiltrate in acute rejection. **b** In a liver graft with rejection and concomitant CMV infection, COX-2-positive cells are seen in the sinusoids and between the hepatocytes (**a** and **b**: original magnification ×400, paraffin-embedded tissue sections). **c** A higher-power view of a COX-2-positive cell in the sinusoidal area. Localization and morphology are quite similar to **d** ED2-positive cells, although the number of ED2-positive cells is higher (**c** and **d**: original magnification ×1,000, **c** paraffin-embedded tissue section, **d** frozen section)

the uninfected group, there was significant increase in the number of COX-2-positive cells in the parenchymal/sinusoidal area of the liver.

The relative number of COX-2-positive mononuclear cells in the portal area was actually lower in acute rejection, due to the massive increase in total mononuclear cell count. Also, the effect of CMV on sinusoidal COX-2 expression at day 7 was not significant when expressed in relation to the total mononuclear cell number. There were significantly more mononuclear inflammatory cells in the sinusoidal area in the CMV group on day 7, and this could explain the difference. However, at 4 weeks, the significant increase in sinusoidal COX-2 expression in the CMV group was seen also in the relative numbers, since the total amounts of infiltrating cells were similar. Acute liver rejection is characterized by massive infiltration of the portal tracts by mononuclear inflammatory cells. Most of the increase is accounted for by lymphocytes, while COX-2-expressing cells were probably of the monocyte/macrophage lineage. Therefore, the mere percentage of COX-2-positive cells of all mononuclear cells may also be misleading.

It is not clear why the site of induction of COX-2 is different in the CMV-infected and the uninfected group. The site of rat CMV infection in our allografts does not explain why the increase in COX-2 expression by rat CMV was seen in the sinusoidal area, since we have previously demonstrated rat CMV antigens in both portal and parenchymal areas during the infection [13]. Neither was the difference explained by the numbers of macrophages/Kupffer cells in the two groups. We have previously shown that expression of VCAM-1 is up-regulated by CMV in the sinusoidal endothelium in this model [13]. VCAM-1 has been shown to be important for monocyte extravasation [9]. An additional explanation for CMV-induced COX-2 expression in the sinusoids would be Kupffer-cell activation as a general physiological response to viral infection.

In vitro-studies indicate that CMV stimulates arachidonic acid metabolism [1] and that inhibitors of prostaglandin synthesis inhibit growth of CMV [21, 22]. In one study, however, rat CMV infection was shown to decrease the number of arachidonic acid metabolites in peritoneal macrophages [6]. CMV immediate early proteins have been shown to transactivate the COX-2 gene, probably via NF-κB, and COX-2 has been reported to play an important role in the cell activation cascade induced by CMV, making the environment favorable for replication of the virus [21]. This activation includes up-regulation of pro-inflammatory genes, and therefore COX-2 could be involved in the association of CMV and allograft rejection. In rat cardiac allograft rejection, COX-2 was shown to be up-regulated, and COX-2 inhibition led to modest but significant prolongation of graft survival [23], indicating that COX-2 is not necessary for acute rejection, but that its action can be replaced by other factors. On the other hand, COX-2 has also been associated with immunomodulatory, possibly tolerogenic, signals [16, 17], and prostaglandins have also been suggested to mediate the immunosuppressive effect of CMV [16].

In conclusion, in our model, rejection up-regulates COX-2, but concomitant CMV infection is still able to increase significantly the number of COX-2-positive cells

in the graft. Our results support the importance of COX-2 for CMV and a role for COX-2 in the association of CMV infection with allograft rejection. Further studies with specific COX-2 inhibitors are needed to confirm this hypothesis.

Acknowledgements We thank Tuula Stjernwall, of the Finnish Occupational Health Institute, for her valuable technical advice concerning the immunohistochemistry; Tarja Markov, Raisa Loginov, Miia Mäkijouppila, and Stephen Venn for excellent technical assistance; and Kari Savelius for animal care. This study was supported by grants from the University of Helsinki, Helsinki University Hospital Research Funds (EVO), the Biomedicum Helsinki Foundation, and the Sigrid Juselius Foundation.

References

1. AbuBakar S, Boldogh I, Albrecht T (1990) Human cytomegalovirus stimulates arachidonic acid metabolism through pathways that are affected by inhibitors of phospholipase A2 and protein kinase C. Biochem Biophys Res Commun 166:953–959
2. Arnold JC, Portmann BC, O'Grady JG, Naoumov NV, Alexander GJM, Williams R (1992) Cytomegalovirus infection persists in the liver graft in the vanishing bile duct syndrome. Hepatology 16:285–292
3. Bruggeman CA, Meijer H, Dormans PHJ, Debie WHM, Grauls GELM, van Boven CPA (1982) Isolation of a cytomegalovirus-like agent from wild rats. Arch Virol 73:231–241
4. Bruggeman C, Meijer H, Bosman F, van Boven C (1985) Biology of rat cytomegalovirus infection. Intervirology 24:1–10
5. Cakaloglu Y, Devlin J, O'Grady J, Sutherland S, Portmann BC, Heaton N, Tan KC, Williams R (1995) Importance of concomitant viral infection during late acute liver allograft rejection. Transplantation 59:40–45
6. Engels W, Grauls G, Lemmens PJ, Mullers WJ, Bruggeman CA (1989) Influence of a cytomegalovirus infection on functions and arachidonic acid metabolism of rat peritoneal macrophages. J Leukoc Biol 45:466–473
7. Garcia-Valdecasas JC, Rull R, Grande L, Fuster J, Rimola A, Lacy AM, Gonzalez FX, Cugat E, Puig-Parellada P, Visa J (1995) Prostacyclin, thromboxane, and oxygen free radicals and postoperative liver function in human liver. Transplantation 60:662–667
8. Geist LJ, Hopkins HA, Dai LY, He B, Monick MM, Hunninghake GW (1997) Cytomegalovirus modulates transcription factors necessary for the activation of tumor necrosis factor-α promoter. Am J Respir Cell Mol Biol 16:31–37
9. Gerszten RE, Lim YC, Ding HT, Snapp K, Kansas G, Dichek DA, Cabanas C, Sanchez-Madrid F, Gimbrone MA Jr, Rosenzweig A, Luscinskas FW (1998) Adhesion of monocytes to vascular cell adhesion molecule-1-transduced human endothelial cells: implications for atherogenesis. Circ Res 82:871–878
10. Kamada N, Calne RY (1979) Orthotopic liver transplantation in the rat. Technique using cuff for portal vein anastomosis and biliary drainage. Transplantation 28:47–50
11. Lautenschlager I, Höckerstedt K, Jalanko H, Loginov R, Salmela K, Taskinen E, Ahonen J (1997) Persistent CMV in liver allografts ending up with chronic rejection. Hepatology 25:190–194
12. Martelius T, Mäkisalo H, Höckerstedt K, Taskinen E, Lautenschlager I (1997) A rat model of monitoring liver allograft rejection. Transpl Int 10:103–108
13. Martelius T, Krogerus L, Höckerstedt K, Bruggeman C, Lautenschlager I (1998) Cytomegalovirus infection is associated with increased inflammation and severe bile duct damage in rat liver allografts. Hepatology 27:996–1002
14. Mitchell JA, Larkin S, Williams TJ (1995) Cyclo-oxygenase-2: regulation and relevance in inflammation. Biochem Pharmacol 50:1535–1542
15. Moran M, Mozes MF, Maddux MS, Veremis S, Bartkus C, Ketel B, Pollak R, Wallemark C, Jonasson O (1990) Prevention of acute graft rejection by the prostaglandin E1 analogue misoprostol in renal-transplant recipients treated with cyclosporine and prednisone. N Engl J Med 322:1183–1188
16. Nokta MA, Hassan MI, Loesch K, Pollard RB (1996) Human cytomegalovirus-induced immunosuppression. Relationship to tumor necrosis fac tor-dependent release of arachidonic acid and prostaglandin E2 in human monocytes. J Clin Invest 97:2635–2641
17. Perez RV, Swanson C, Morgan M, Erickson K, Hubbard NE, German JB (1997) Portal venous transfusion upregulates Kupffer cell cyclo-oxygenase activity: a mechanism of immunosuppression in organ transplantation. Transplantation 64:135–139
18. Post S, Goerig M, Otto G, Manner M, Foltis C, Hofmann W, Herfarth C (1991) Rapid increase in the activity of enzymes of eicosanoid synthesis in hepatic and extrahepatic tissues after experimental liver transplantation. Transplantation 51:1058–1065
19. Ruiz P, Coffman TM, Klotman PE, Sanfilippo F (1989) Association of chronic thromboxane inhibition with reduced in situ cytotoxic T cell activity in rejecting rat renal allografts. Transplantation 48:660–666
20. Speir E, Shibutani T, Yu ZX, Ferrans V, Epstein SE (1996) Role of reactive oxygen intermediates in cytomegalovirus gene expression and in the response of human smooth muscle cells to viral infection. Circ Res 79:1143–1152
21. Speir E, Yu ZX, Ferrans VJ, Huang ES, Epstein SE (1998) Aspirin attenuates cytomegalovirus infectivity and gene expression mediated by cyclo-oxygenase-2 in coronary artery smooth muscle cells. Circ Res 83:210–216
22. Tanaka J, Ogura T, Iida H, Sato H, Hatano M (1988) Inhibitors of prostaglandin synthesis inhibit growth of human cytomegalovirus and reactivation of latent virus in a productively and latently infected human cell line. Virology 163:205–208
23. Yang X, Ma N, Szabolcs MJ, Zhong J, Athan E, Sciacca RR, Michler RE, Anderson GD, Wiese JF, Leahy KM, Gregory S, Cannon PJ (2000) Up-regulation of COX-2 during cardiac allograft rejection. Circulation 101:430–438