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Chronic Myeloproliferative Disorders

Expression of polycythemia rubra vera-1 decreases the dependency of cells on growth factors for proliferation

An increase in the level of polycythemia rubra vera -1 (PRV-1) mRNA has been reported in some myeloproliferative disorders. We have studied the effects of PRV-1 on cell proliferation and cell survival. In cell growth assays, the number of heterologous cells expressing PRV-1 increased faster than sham-transfected cells, a difference that was more pronounced in serum-free media. Even after 5 days of exposure to serum-free media, cells expressing PRV-1 continued to proliferate, whereas the control cells ceased to proliferate. We conclude that PRV-1 is a pro-proliferation molecule, and hypothesize that its overexpression may have a role in the pathogenesis of myeloproliferative disorders.

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Polycythemia rubra vera-1 (PRV-1, also known as CD177 or NB1) is a member of Ly-6/uPAR protein family, whose members have been shown to play a role in cell proliferation.[1] In normal subjects, PRV-1 is expressed on a subpopulation of neutrophils, on myelocytes and on metamyelocytes. An increase in the level of mRNA encoding PRV-1 has been reported in neutrophils from patients with polycythemia vera (PV) and essential thrombocythemia (ET);[2-6] however, the function of PRV-1 is unknown. We have conducted *in vitro* studies investigating the function of PRV-1 in a heterologous cell line stably expressing this molecule. After transfecting either PRV-1 cDNA or empty plasmid to Chinese hamster ovary (CHO) cells, we established a stable CHO cell line expressing PRV-1 (CHO-PRV-1) and a control cell line (CHO-Sham). Flow cytometry studies with MEM-166, a monoclonal antibody to CD177 (BD Pharmingen, San Diego, CA, USA), showed that CHO-PRV-1 cells, but not CHO-Sham cells, expressed PRV-1 (*data not shown*). In the cell growth assay, the number of CHO-PRV-1 cells increased faster than CHO-Sham cells in the presence of 10% fetal bovine serum (FBS, Sigma-Aldrich) in the growth media (Figure 1A).

The difference in the number of cells was statistically significant at each time-point, except at 0 and 24 hr, and persisted throughout the 5-day follow-up. This difference in growth rates between CHO-PRV-1 and CHO-Sham cells was further increased when the cells were grown in the absence of FBS. The growth curves diverged further with time, and the difference in the cell number was most prominent on the fifth day (Figure 1B).

We studied cell proliferation by measuring the percentage of bromodeoxyuridine (BrdU)-incorporating cells. On the first day after exposure to serum-free media, the percentage of BrdU (+) cells was similar between CHO-PRV-1 and CHO-Sham cells; however, starting on the 4th day, there was a significantly higher percentage of proliferating cells among CHO-PRV-1 cells than among control cells (8.8±0.8% vs 0.8±0.1%, respectively; p=0.02) (Figure 2A). Additionally, we compared the rate of apoptosis and necrosis between the two cell lines by measuring binding of annexin V and incorporation of SYTOX green stain, using the Vybrant Apoptosis Assay Kit (Molecular Probes, Eugene, OR, USA). We found that although apoptosis was not different between CHO-PRV-1 and CHO-sham cells (Figure 2B), the percentage of necrotic cells was higher in sham-transfected cells than in PRV-1-expressing cells (Figure 2C).

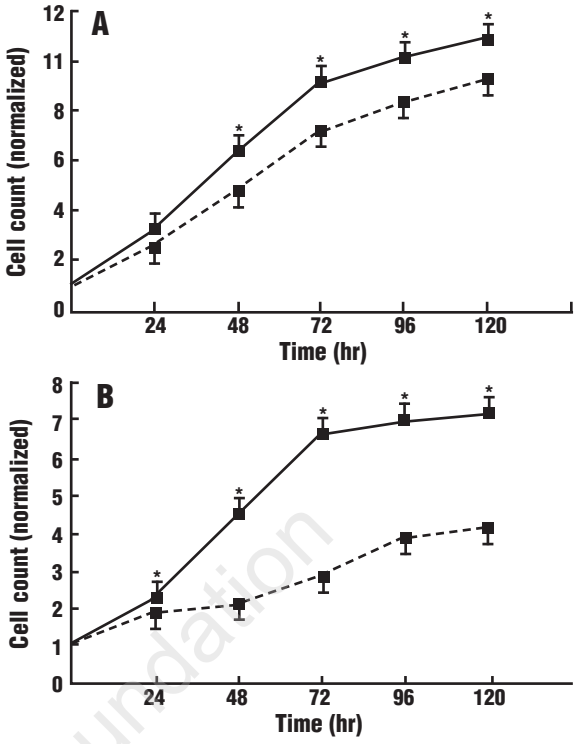

Figure 1. Cell growth and expression of PRV-1: 1×10^9 CHO-PRV-1 (-■-) or CHO-Sham cells (--■--) were seeded in 6–well plates. Four to 6 hours after seeding (time 0), growth media were replaced with DMEM (Invitrogen) with either 10% FBS (a) or no FBS (b). At 24-hour intervals, cells were detached from the plates and counted with a hemocytometer. The figure summarizes the results of 3 separate experiments. The number of cells at any time point was normalized to the initial cell count and is shown on the Y-axis (Cell count at each time-point/Cell count at 0 time-point). *Shows statistically significant differences in the cell counts (p<0.05, Student's t-test).

There are clinical observations that support a pro-proliferative role for PRV-1. Clinical settings that are associated with elevated PRV-1 expression, such as administration of granulocyte colony-stimulating factor, sepsis, pregnancy, and polycythemia vera are also known to be associated with higher neutrophil counts.[7-9]

Another finding of our studies is that expression of PRV-1 reduces the dependency of cell proliferation on growth factors present in serum. Interestingly, in a recent study, only progenitor cells of ET patients whose neutrophils overexpressed PRV-1 were capable of forming *in vitro* colonies in the absence of erythropoietin.[10] Several important questions on the expression of PRV-1 are unanswered. Do early hematopoietic progenitor cells

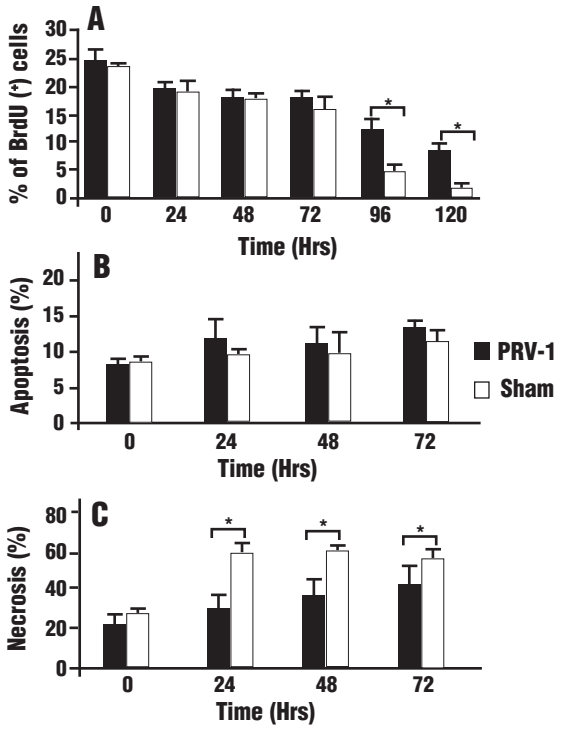

Figure 2. Effect of PRV-1 expression on cell proliferation and cell death. A. *Cell proliferation*: equal numbers of transfected CHO cells (1x10⁴) were seeded in 4-well Laboratory Teck chamber slides (Nalge Nunc International, Rochester, NY, USA). Cells were serum-starved for 0, 24, 48, 72, 96, and 120 hours; pulsed with 50 mol/L BrdU, and then fixed in 70% ethanol solution at -20°C. The Y-axis shows the percentage of BrdU-positive cells [BrdU-positive cells/total cell count) × 100]. BrdU incorporation into the nuclei of cells was evaluated by light microscopy at 20 X magnification and for each time point, a total of 1,000 cells was counted. B-C. *Cell death*: equal numbers of transfected CHO-cells were serum starved for different time intervals. We used flow cytometry to measure the surface binding of annexin V (apoptotic cells) or DNA incorporation of SYTOX green dye together with surface binding of annexin V (necrotic cells). Percentages of apoptotic (B) or necrotic (C) cells are shown on the X-axis. The experiment was repeated three times and the results are summarized as bar graphs *shows $p<0.05$ (Student's t-test).

express PRV-1 mRNA? Is there a difference in the expression of PRV-1 on hematopoietic progenitor cells between normal subjects and patients with myeloproliferative diseases ? Is there a correlation between the level of mRNA and surface expression of PRV-1? There are controversial data about the answer to this last question.[4,7] Besides being a possible diagnostic marker for PV and ET, PRV-1 overexpression may alter cellular function. We found that expression of PRV-1 on CHO cells decreased the dependency of these cells on serum for survival and proliferation. The role of PRV-1 in proliferation of myeloid cells and in the pathogenesis of PV and ET should be studied further in other *in vitro* systems and in animal models.

Zakar Mnjoyan, Jun Li, Vahid Afshar-Kharghan

Thrombosis Research Section, Baylor College of Medicine, Houston, TX, USA

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Key words: myeloproliferative disorders, PRV-1, cell proliferation, growth factors.

Correspondence: Vahid Afshar-Kharghan, M.D., Thrombosis Research Section, Department of Medicine, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. Phone: international +1.713.7982955. Fax: international +1.713.7983415. E-mail: vahid @bcm.tmc.edu

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Chronic Myeloproliferative Disorders

Neutrophil polycythemia rubra vera-1 expression in classic and atypical myeloproliferative disorders and laboratory correlates

The current study of 153 subjects with both classic and atypical myeloproliferative disorders suggests that neutrophil polycythemia rubra vera-1 (PRV-1) over-expression is a non-specific feature of clonal myeloproliferation that displays significant correlation with leukocyte alkaline phosphatase score. These observations undermine the utility of the PRV-1 assay as a diagnostic test of additional value.

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Previous studies have found a strong association between neutrophil PRV-1 over-expression and polycythemia vera (PV) which is neither invariable (test sensitivity ranging from 69-100%)[1-6] nor exclusive (a substantial minority of patients with either essential throm-