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Transcription factors down-stream of Ras as molecular indicators for targeting malignancies with oncolytic herpes virus

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ABSTRACT

Overactivation in Ras signaling has been under intensive study as the molecular basis for development of cancer. Such overactivation can occur in the presence or absence of mutations in Ras gene resulting in activation of a series of down-stream effectors such as transcription factors. Different studies have shown the activation of Ras down-stream effectors in non-Hodgkin lymphoma (NHL) although mutations in Ras are not prevalent in this malignancy. Since overactivation in Ras signaling also increases permissiveness of cancer cells to infection by oncolytic versions of herpes simplex virus (e.g. R3616), we were interested in evaluating the value of transcription factors down-stream of Ras as molecular indicators for permissiveness to herpes therapy. In order to accomplish this, and also to assess the permissiveness of lymphoma cells to infection with R3616, we used NHL cell lines Daudi, Jurkat, NC37, Raji, Ramos and ST486. Once the levels of phosphorylation (activation) of extracellular-signal regulated kinase (ERK, a Ras effector pathway) and its down-stream transcription factor ELK were evaluated, Raji and NC37 showed a significant increase in the phosphorylation levels of both molecules while ATF2 (another transcription factor down-stream of p38-kinase pathway) seemed to be activated in all studied cells. Raji and NC37 cells were also most permissive cells to infection with R3616 while their permissiveness was decreased upon treatment of cells with an inhibitor of ELK-DNA binding portraying ERK/ELK as a suitable predictive indicator for selection of cancer cells with increased sensitivity to R3616. This study, therefore, for the first time documents permissiveness of lymphoma cells to oncolytic herpes viruses and introduces ELK as a suitable factor for predicting tumor susceptibility to these novel anticancer agents.

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1. Introduction

An interesting aspect of interaction between cells and viruses has recently emerged from studies revealing the potentials of some viruses for targeting cancer cells. Oncolytic viruses are novel therapeutic agents being investigated for their efficacy against different types of human tumors in pre-clinical and clinical trials (Parato et al., 2005). We have previously shown that overactivation in Ras signaling increases permissiveness of cells to infection by herpes simplex virus-1 (HSV-1) and oncolytic mutant version of it, R3616, which bears deletion in both copies of viral γ_1 34.5 gene (Farassati and Lee, 2003; Farassati et al., 2001). Although mutations in N- and K-Ras exist in about 40% of myelodysplastic syndrome and 15% of AIDS-related NHL cases (Downward, 2003), an expanding body of evidence is now referring to the activation of down-stream Ras signaling such as extracellular regulated kinase (ERK) and p38-kinase pathway in lymphoma (Jazirehi et al., 2004; Kawauchi et al., 2002; Kurland et al., 2003; Ogasawara et al., 2003; Roberts and Cooper, 1998; Wilson et al., 1999). It is therefore reasonable to expect that transcription factors down-stream of Ras, such as ELK and ATF2, would be elevated in some cases of lymphoma or other hematological malignancies, where a constitutive activation of up-stream players such as Ras/ERK exists. Therefore, we were interested in assessing the permissiveness of lymphoma cells to infection with oncolytic herpes. We also evaluated the value of transcription factors down-stream of Ras as molecular indicators for permissiveness to herpes therapy.

2. Design and method

Human lymphoma cell lines were obtained from American type culture collection and maintained in RPMI-10% fetal bovine serum, 1% L-glutamine, and antibiotics. Stock solutions of Chromomycin A₃ were prepared in water and DMSO and stored in -20°C . R3616, was a generous gift from Dr. Bernard Roizman. The growth, plaque titration and stock preparation were performed as explained elsewhere (Brown and Maclean, 1998). Expression of herpetic proteins was detected by using an antibody raised against all HSV-1 antigens (DAKO, CA) as explained in our previous work (Farassati et al., 2001). Antibodies against total and phosphorylated forms of ELK and ATF2 were obtained from Millipore (MA). Antibodies against total and phosphorylated forms of ERK were obtained from Cell Signaling (MA). A total amount of 100 μg of NHL cell lysates was loaded for detection of transcription factors (ELK and ATF2) and 50 μg for ERK.

3. Results and discussion

Ras signaling was assessed by immunoblotting for different down-stream effectors such as ERK, ELK and ATF2 (the last two function as transcription factors). While active-ERK (phospho-ERK) somewhat existed in Daudi, Raji and Jurkat cells, its levels were higher in Raji (Figure 1A). Among another three NHL cell lines, NC37 contained higher phospho-ERK as

compared with ST486 and Ramos. Our investigation of the ELK activation, however, produced a more definitive portfolio in which the only cells with detectable levels of phospho-ELK were Raji and NC37 (Figure 1B). Phospho-ATF2, a transcription factor mainly acting down-stream of p38-kinase pathway seemed to be relatively active in all studied NHL cell lines (Figure 1C). In our previous studies we have found that, ERK pathway, but not p38-kinase pathway, was involved in herpes infection (Farassati and Lee, 2003; Farassati et al., 2008, 2001). So here we tried to once again study this matter by investigating the role of ELK (a major down-stream substrate of ERK pathway) and ATF2 (a major down-stream substrate of p38-kinase pathway). The prediction was that while infectibility by R3616 would have a correlation with the levels of ELK phosphorylation, phospho-ATF2 levels will not be a good predictor of viral infection. Such prediction was proven to be accurate on the basis of the data explained above.

In next step, we evaluated the permissiveness of NHL cells to R3616. We first compared Burkitt lymphoma derived cell lines Raji and Daudi and T-cell lymphoma cell line Jurkat by western blotting for herpes proteins at 24 h post-infection. Following infection with R3616 at MOI~1 pfu/cell, the highest level of viral protein synthesis was observed in Raji, while no viral protein synthesis was detectable in Daudi or Jurkat cells (Figure 1D). Similarly, at MOI~5 and 10, although an increase in herpetic proteins was observed, Raji cells remained the top producer of viral proteins. We also infected the same cells with R3616 parental virus, HSV-1 (strain F, MOI~1) and ascertained that efficient viral protein synthesis took place only in Raji cells. This was taken as one level of evidence for increased permissiveness of Raji cells to R3616 infection. In order to verify this, viral progeny titration at 24–48 h post-infection with R3616 was also evaluated. At 24 h, the titration for viral progeny for Raji cells was 7.8×10^6 pfu/ml, Daudi at 1.7×10^6 pfu/ml and least susceptible Jurkat indicated only 1.5×10^6 pfu/ml (Figure 1E). At 48 h, Raji achieved over 14×10^6 pfu/ml, unlike Daudi at 4×10^6 pfu/ml and Jurkat at 3×10^6 pfu/ml. Resembling data was found for NC37, ST486 and Ramos cells where NC37 exhibited increased level of viral progeny after R3616 infection (data not shown).

Therefore, in harmony with our previous findings, Raji and NC37 cells with elevated levels of ERK and ELK activation seem to be most permissive to infection with R3616. Such observation also indicates that transcription factor ELK may be a more suitable predictive factor for selection of highly permissive cells since Raji and NC37, the only cells with detectable amounts of phospho-ELK, were also most infectible by R3616. ATF2, however, did not show any relevance to infectibility by R3616 since it was found to be phosphorylated in all studied cells. Such conclusion is also in agreement with our previous studies in which we found that p38-kinase pathway (the main up-stream activation pathway for ATF2) is not involved in enhanced permissiveness of Ras transformed cells to herpes (Farassati et al., 2001).

In order to further expand our observation on the role of the transcription factor ELK in permissiveness of cells to R3616 we used Chromomycin A₃, a drug with ELK-DNA inhibiting properties (White et al., 2000) which reduces ELK's

Figure 1 – Ras down-stream signaling and permissiveness to herpes virus in NHL cells (A) the activity of Ras/ERK pathways was determined in NHL cells by blotting for phosphorylated (activated) form of ERK. The total form of this enzyme was also evaluated by using an anti-ERK antibody (B) the level of activation of ELK was evaluated by blotting for the phosphorylated (activated) form of ELK. The lower panel shows the total amount of ELK in NHL cells (C) the activated and total forms of the transcription factor ATF2 were evaluated by blotting cell lysates with antibodies specific for activated (phosphorylated) or total forms of ATF2 (D) viral protein synthesis in Daudi, Raji and Jurkat cells after infection of these cells with R3616 or HSV-1 (F) with different multiplicities of infection (MOI) ranging from 1 to 10 for R3616 and 1 for HSV-1 at 24-h post-exposure to viruses. A significantly increased amount of protein synthesis is observed for Raji cells. Control (uninfected) cell lines confirm the specificity of the antibody for herpetic proteins (E) viral progeny titration in Daudi, Raji and Jurkat cells after infection of these cells with R3616 at MOI ~2. In Raji 7.8×10^6 pfu/ml, Daudi 1.7×10^6 pfu/ml and Jurkat 1.5×10^6 pfu/ml. At 48 h: Raji 14×10^6 pfu/ml, Daudi 4×10^6 pfu/ml and Jurkat 3×10^6 pfu/ml. Cell designations are: D = Daudi, R = Raji, J = Jurkat, N = NC37, S = ST486 and R = Ramos.

Figure 2 – The effects of inhibition of Ras down-stream transcription factors on permissiveness of NHL cells to R3616 (A) schematic representation of protein complex recruited to the site for serum response element including the site of action for ELK transcription factor (B) chemical structure of Chromomycin A₃ (C) the effects of treatment of NC37 cells with Chromomycin A₃ (750 picogram) on progeny virus production at 24 and 48 h post-infection with R3616. Cells were treated with Chromomycin over night and then exposed to virus (MOI ~ 2) for 24–48 h in serum containing media (D) the effects of treatment of NC37 cells with Chromomycin A₃ (750 picogram) on viral protein synthesis at 24 and 48 h post-infection with R3616. Cells were treated in the same manner as (C) and the lysates were collected at 24-h post-infection with R3616.

transcriptional activity (Figure 2A and B). Chromomycin A₃ is a minor groove binding agent which binds to G/C rich areas of SRE. Binding of this agent to SRE inhibits formation of the ternary complex. Cell free transcription and endogenous C-fos expression in NIH3T3 cells have proven the ability of

Chromomycin A₃ in inhibiting the transcriptional activity of ELK (White et al., 2000). Following treatment of NC37 cells with Chromomycin (0.750 nM, overnight) and infecting them with R3616 for 24 h (MOI ~ 2), these cells showed a decrease in the viral protein synthesis (Figure 2C). Expectedly, they

also showed a reduction in the progeny virus titration from 6×10^6 to 2×10^6 pfu/ml (Figure 2D). Such inhibitory effects existed at 48 h post-infection.

The research on treating human malignancies with oncolytic viruses has led us to the need for markers which can be used for selection of responsive neoplasms. While other studies have shown the efficiency of other oncolytic viruses in treatment of lymphomas in pre-clinical studies (Alain et al., 2002) we have focused on signaling down-stream of Ras such as ERK, ELK and ATF2 which might play a role in determining the infectibility of NHL cells by oncolytic herpes. ELK, in this regard, was found to be a stringent molecular indicator for permissiveness to R3616. We also assume that cellular transcriptional events down-stream of ELK might be important for infection since blockade of ELK function (by Chromomycin) reduces permissiveness of NC37 cells.

Previous studies have also confirmed dependence of another oncolytic virus, reovirus, on Ras signaling machinery for infection (Alain et al., 2002). Indeed, studies of tumor regression *in-vivo*, show that the Raji derived tumor growth in live virus-treated mice was inhibited as compared with control mice treated with UV-inactivated virus (Alain et al., 2002). In contrast to this, Daudi tumors were resistant to this therapy. These results remain in agreement with our *in-vitro* studies by means of herpes viruses; moreover the step forward in our experiments is the elucidation of signaling molecules that have predictive role for such preferential permissiveness. In this study, we managed to deliver evidence for the importance of Ras down-stream effectors pathways in determining the permissiveness of NHL to R3616. The possibility of using this knowledge to foresee the suitability of viral anticancer therapy opens a new horizon for application of these novel agents.

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Appendix. Supplementary data.

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.molonc.2009.07.002](https://doi.org/10.1016/j.molonc.2009.07.002).

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