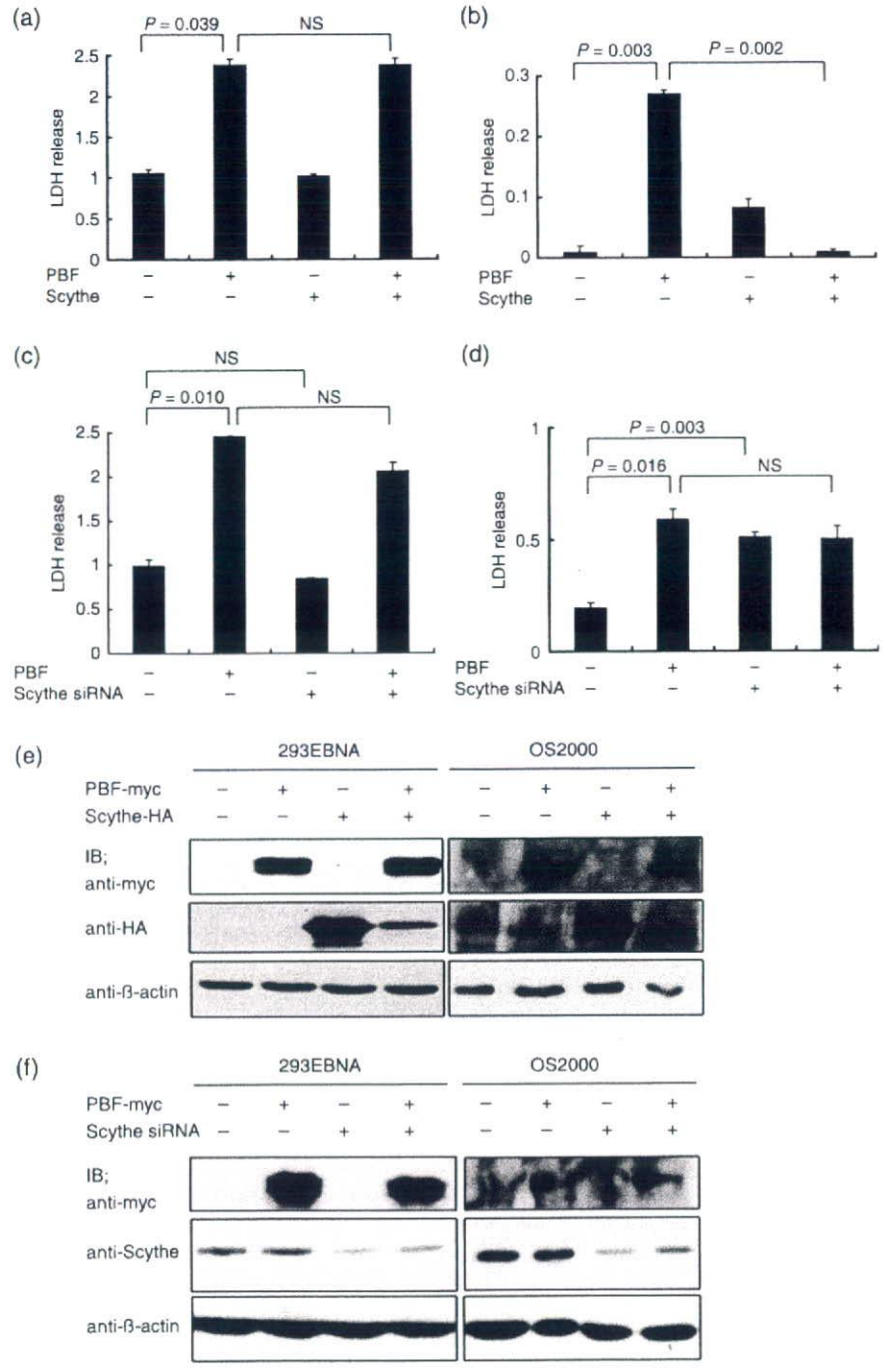

Fig. 6. Effects of papillomavirus binding factor (PBF) and Scythe/BAT3 on apoptotic cell death. (a) 293EBNA or (b) OS2000 cells were transfected with PBF-myc/pCMV, Scythe/BAT3-HA/pCMV, or both plasmids. pCMV was used for mock transfection. LDH release into the supernatant from transfected cells (resulting from cell death) was assessed. (c) 293EBNA or (d) OS2000 cells were transfected with PBF-myc/pCMV, small interfering RNA (siRNA) silencing Scythe/BAT3 or PBF-myc/pCMV and Scythe/BAT3-HA/pCMV PCMV and control siRNA were used for mock transfection. (e) Transfected PBF-myc and Scythe/BAT3-HA and (f) endogenous Scythe/BAT3 that was downregulated by siRNA in 293EBNA and OS2000 cells were detected. NS, not significant

(i) PBF expression in the nuclei of OS2000 cells could induce apoptosis, like PBF in the cytoplasm of 293EBNA cells; (ii) nuclear PBF-induced apoptosis in OS2000 cells could be suppressed by colocalization of Scythe/BAT3; and (iii) in contrast, cytoplasmic PBF-induced apoptosis in 293EBNA cells could not be suppressed by colocalization of Scythe/BAT3. Taken together, these results suggest that colocalization of PBF and Scythe/BAT3 in the nucleus, which was observed in OS2000 cells, could regulate PBF-induced cell death and might be an important factor in the survival of osteosarcoma cells.

Discussion

In the present study, we demonstrated that: (i) overexpressed PBF could induce apoptotic cell death via a caspase-9-independent pathway; (ii) Scythe/BAT3 was identified as a molecule associated with PBF after screening of a cDNA library of OS2000 using a yeast two-hybrid system; (iii) Scythe/BAT3 mRNA was detected in 56% of osteosarcoma primary tissues and ubiquitously in various normal organs; (iv) Scythe/BAT3 protein was localized to the nuclei of osteosarcoma tissue but the cytoplasm of normal tissues; and (v) PBF-induced apoptotic cell death was suppressed by colocalization of Scythe/BAT3 with PBF in the nuclei of OS2000 but not in the cytoplasm of 293 cells. These findings suggest that Scythe/BAT3 might play a role in regulating PBF-mediated apoptosis and that the proliferation of osteosarcoma cells is also regulated by PBF and Scythe/BAT3 that colocalize to the nucleus.

Papillomavirus binding factor has been identified as a transcription factor of a promotor in the human papillomavirus type

8 genome,[1] a Huntington's disease-associated protein,[20] and a tumor-associated antigen overexpressed in various sarcomas and carcinomas.[2,21] Thereafter, Sichtig *et al.* reported that overexpression of PBF in the cytoplasm induces cell death and the subcellular localization of PBF is regulated by 14-3-3, which is a cytoplasmic protein, and could promote cell survival in a skin keratinocyte cell line.[11,22] We also confirmed that overexpression of PDF was localized in cytoplasm of 293 cells and induces cell death, as shown in our current study.

Generally, endogenous cell stress, which is evoked by subcellular expression of apoptotic molecules, induces the intrinsic mitochondrial pathway for apoptosis induction, including the mitochondrial release of cytochrome *c*, which binds to apoptosis protease-activating factor-1. This complex, which is called the apoptosome, activates caspase-9, triggering downstream caspase-3.[5,8] However, as shown in our current study, the overexpression of PBF induces apoptosis, indicated by caspase-3 activity and cleavage of PARP, and resulted in cell death, but did not activate caspase-9. This suggests that overexpression of PBF induces apoptosis via a caspase-9-independent pathway. Therefore, we screened PBF-binding molecules using a yeast two-hybrid system to identify apoptotic regulators of PBF, and isolated Scythe/BAT3.

Scythe has been identified as a nuclear protein regulating apoptosis, associated with Reaper in *Drosophila melanogaster*,[15] and BAT3 has been identified as a human homolog of Scythe.[16] Scythe/BAT3 is required for acetylation of p53 in response to DNA damage[23] and drug-induced apoptosis.[18] However, Scythe/BAT3 was reported to be an anti-apoptotic protein.[24,25] Scythe/BAT3 is essential for cell proliferation,[18] can suppress apoptotic activity of the *Xenopus laevis* elongation factor 1α oocyte form (XEF1AO), and might control development in *Xenopus* embryos.[26] In addition, Scythe/BAT3 contains a Bcl-2-associated athanogene (BAG) domain at the C-terminus and inhibits heat shock protein 70-mediated protein refolding.[27] In fact, Scythe/BAT3 and heat shock protein 70 are cochaperones of human small glutamine-rich tetratricopeptide repeat (TRP)-containing protein, which is an essential molecule for cell division.[28] However, the functions of Scythe/BAT3 in tumor cells remain unknown.

In the present study, PBF-induced cell death was suppressed by overexpressed Scythe/BAT3 in nuclei of osteosarcoma cells. However, overexpressed Scythe/BAT3 localized to the cytoplasm did not suppress PBF-induced cell death in spite of colocalization of PBF and Scythe/BAT3. These findings suggest that overexpression of Scythe/BAT3 functions as an anti-apoptotic factor in tumor cells. Expression of Scythe/BAT3 at the endogenous normal level is required for signaling apoptosis but its overexpression induces an anti-apoptotic effect via upregulation of Bcl-2.[24] Recently, Scythe/BAT3 was shown to stabilize and interact with AIF,[29] which can lead to chromatin condensation and DNA fragmentation by its relocation from the mitochondria to the nucleus.[10] In severe DNA damage, PARP-1 activation triggers AIF translocation from mitochondria to the nucleus, followed by mitochondrial depolarization, release of cytochrome *c*, and activation of caspase-3, which cleaves PARP.[30,31] Moreover, overexpression of Bcl-2, which is upregulated by overexpression of Scythe/BAT3,[24] also suppresses AIF release from mitochondria.[30] Thus, it appears that overexpression of PBF might induce apoptotic cell death via PARP-1 and an AIF-dependent pathway, followed by mitochondrial release of caspase-3 cleaving PARP-1. Then Scythe/BAT3-mediated suppression of PBF-induced cell death might result from inhibition of AIF translocation to the nucleus by Bcl-2 upregulated via Scythe/BAT3 overexpression.

Regarding whether the normal level of Scythe/BAT3 could act as an anti-apoptotic factor, we observed that silencing of Scythe/BAT3 significantly increased LDH release in OS2000 with the mock control but did not change it in OS2000 with PBF. Although we predicted that silencing of Scythe/BAT3 would increase cell death events induced by PBF, we thought that natural PBF expression might be the reason why there was no difference between OS2000 with the mock control and OS2000 with PBF in the condition of silencing of Scythe/BAT3. However, we also observed that silencing of Scythe/BAT3 did not change LDH release in the case of 293EBNA cells. These results suggest that natural cytoplasmic Scythe/BAT3 could not suppress PBF-induced apoptosis in 293EBNA cells, as was also the case with the overexpressed cytoplasmic Scythe/BAT3.

Our results support the idea that Scythe/BAT3, as well as PBF, could act as a nucleus–cytoplasm shuttling protein regulating apoptosis. Tumor suppressors, such as p53, Forkhead BOX, and phosphatase and tensin homolog, have been reported to shuttle molecules between the nucleus and cytoplasm regulated by mono-ubiquitylation.[32] The localization of these proteins plays a key role in tumor suppression. Nucleus–cytoplasm shuttling of proteins is generally considered an important mechanism for apoptotic regulation.[33,34]

We previously reported that PBF is expressed in the nucleus of primary osteosarcoma tissues and that PBF-positive osteosarcoma and Ewing's sarcoma have significantly poor prognoses.[3,35] Although these findings suggest strongly that PBF regulates the proliferation of osteosaroma cells in humans, it is still unknown how PBF regulates cell death and proliferation *in vivo*. Scythe/BAT3 is a possible factor modulating the function of PBF *in vitro*. Thus, further studies including PBF-depletion *in vitro* and *in vivo* should be considered.

In conclusion, we identified Scythe/BAT3 as a molecule associated with PBF. We have also demonstrated that overexpression of Scythe/BAT3 regulates PBF-induced apoptotic cell death in osteosarcoma cells. Moreover, colocalization of PBF and Scythe/BAT3 in the nucleus might be an important factor for survival of osteosarcoma cells.

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