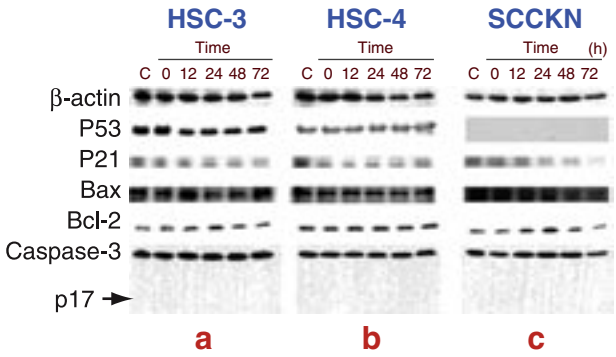

Fig. 6. Western blot analysis of P53, P21, Bax, Bcl-2 and caspase-3 of each cell lines. Cells were infected with Ad.tk at MOI 5 for HSC-3, 2.5 for HSC-4 and SCCKN, and followed by 1 μg/mL GCV. Cells were harvested in a time-dependent manner. Uninfected cells without GCV treatment were used as control. (**a**) HSC-3, (**b**) HSC-4 and (**c**) SCCKN cell lines. C, control.

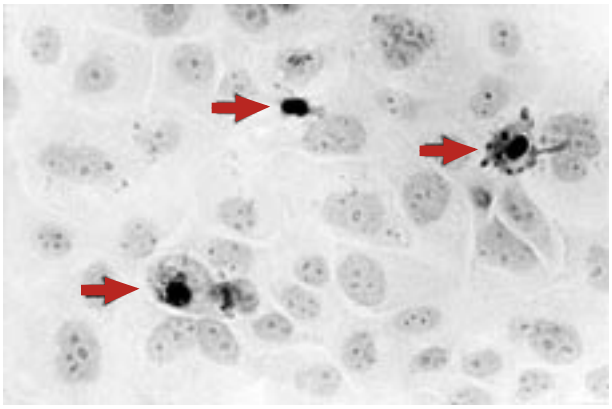

Fig. 7. TUNEL staining of treated SCCKN 24 h after GCV treatment. Cells were infected with Ad.tk at MOI 2.5, and followed by 1 μg/mL GCV. TUNEL-positive cells with brown-labeled nuclei are occasionally detected in only SCCKN cell line (arrows).

in only the SCCKN cell line infected with Ad.tk at MOI 2.5 followed by 1 μg/mL GCV after 24 h (Fig. 7). However, we could not evaluate correctly at 48 h and 72 h because most treated SCCKN cells had easily come off in the staining procedure. However, the untreated SCCKN cells showed no TUNEL signal.

Morphological examinations

There were no significant differences in morphological findings in both semi-thin and ultra-thin sections after HSV-tk/GCV treatment among the cell lines. Semi-thin sections of untreated cells showed variations in size and shape and occasionally small vesicles in the cytoplasm with a few mitoses (Fig. 8a). In contrast, a large number of treated cells became swollen with or without membrane disruption, sometimes involving nuclei disruption (Fig. 8b). Cytoplasmic vesicles were markedly increased. There were few cells with chromatin aggregation in HSC-4 and SCCKN cell lines.

Ultra-thin sections revealed intact nuclei, intracellular organelles and their membranes in untreated cells (Fig. 9a). Otherwise, a large number of treated cells contained enlarged and rounded nuclei in which heterochromatin was sparse. Cytoplasmic membranes were also disrupted (Fig. 9b). A few cells showing chromatin aggregation to the nuclear membranes, also displayed cytoplasmic membranes disruption. Treated cells presented structural changes of intracellular organelles involving enlargement of mitochondria with the disappearance of cristae, vesiculation and vacuolization of endoplasmic reticulum. Apoptotic bodies were not observed in any of the treated cells.

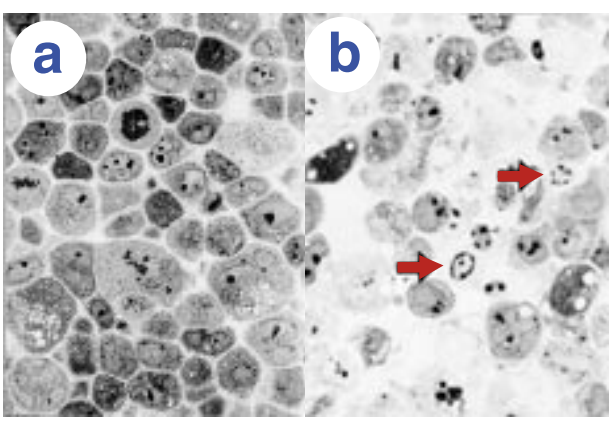

Fig. 8. Semi-thin section micrographs of HSC-4 cells. (**a**) Untreated cells. Some cells occasionally have small vesicles, and some display mitoses. (**b**) Treated cells 72 h after GCV treatment. Cells were infected with Ad.tk at MOI 2.5, and followed by 1 μg/mL GCV. Cells become swollen and intracytoplasmic vesicles remarkably increase. Few cells show chromatin aggregation (arrows).

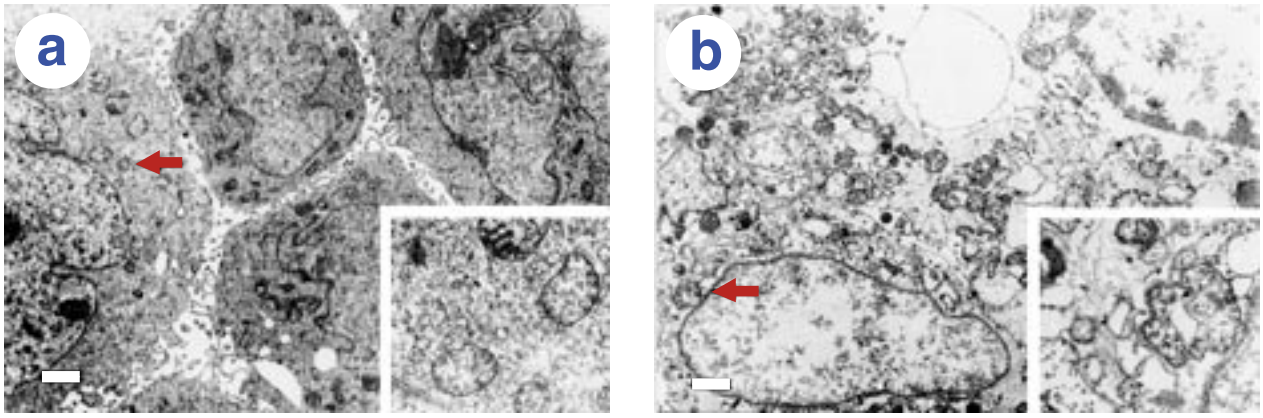

Fig. 9. Ultra-thin section micrograph of HSC-4 cells. (**a**) Untreated cells. (**b**) Treated cells 72 h after GCV treatment. Cells were infected with Ad.tk at MOI 2.5, and followed by 1 μg/mL GCV. A large number of cells contain enlarged and rounded nuclei of which hetero chromatin are sparse. Some of treated cells show chromatin aggregation. But their cytoplasmic membranes are still disruptured. Inset of (**b**) displays enlargement of mitochondria with disappearance of cristae, vesiculation and vacuolization of endoplasmic reticula. Bars in (**a**) and (**b**) = 1 μm.

Discussion

This study clearly demonstrated that HSV-tk/GCV treatment showed diverse but obvious sensitivity to oral SCC cell lines independent of their histological status (Table 1 and Fig. 2). Oral SCC cell lines displayed high susceptibility to HSV-tk/GCV treatment despite low transduction, suggesting possible tumoricidal effects even in the case of inability of 100% gene delivery (Fig. 1). In the HSC-3 cell line, however, HSV-tk/GCV treatment showed the upper limit bounds at MOI 10. As reported previously, a higher expression of HSV-tk gene may not be able to further enhance the tumoricidal effect, though a threshold level of HSV-tk gene expression is necessary for maximum cell killing (Chen et al., 1995).

The tumoricidal mechanism of HSV-tk/GCV treatment has focused on exploring the molecular biological events associated with apoptosis (Samejima and Maruelo, 1995; Wei et al., 1998; Rivas et al., 2001). To date, the most reliable method for identifying apoptotic cell death is the detection of the occurrence of internucleosomal DNA strand breaks (Wyllie, 1980). Concerning past HSV-tk/GCV treatment studies, several reported the existence of DNA laddering (Samejima and Maruelo, 1995; Wei et al., 1998), but several reported the opposite (Kaneko and Tsukamoto, 1995). It is possible that the DNA fragmentation assay might not detect low levels of apoptosis. Confirmation of the phenotype of dying cells is needed by reference to morphology using electron microscopy (Vile et al., 1997). In fact, some cases failed to demonstrate DNA laddering even when the presence of apoptotic cells was further confirmed using electron microscopy (Freeman et al., 1993; Craperi et al., 1999). In the present results, DNA fragmentation showed unspecific DNA degradation, that is, smearing (Fig. 3). Accordingly, both semi- and ultra-thin examinations revealed the phenotype of dying cells was necrotic in all cell lines.

Previous studies of flow cytometry demonstrated that HSV-tk/GCV treatment caused either S- and/or G2/M-phase cell cycle arrest before undergoing cell death (Kaneko et al., 1995; Wei et al., 1998; Craperi et al., 1999). The present results showed cell death progressing with no cell cycle arrest (Fig. 4). The DNA content of sub-G1 increased in a time-dependent manner. A rapid increase was observed in the HSC-4 cell line at 72 h (42.7%), in which DNA fragmentation definitely showed smearing suggesting no occurrence of regular DNA double-strand breaks. Annexin V has been used as another biological marker to detect the early stage of apoptosis before the occurrence

of morphological changes in single cells (Martin et al., 1995). The present results showed that annexin V-positive/PI-negative cells corresponding to apoptotic cells, increased in a time-dependent manner in all cell lines (Fig. 5b), although further semi- and ultra-thin examinations revealed necrotic morphological features. While the externalization of phosphatidylserine during apoptosis has been presented, intracellular events have not been adequately elucidated. Although the meaning of the annexin V-positive reaction induced by HSV-tk/GCV treatment is not contested, this phenomenon implies the feasibility of inspection and exemplification by phagocytic cells (Depraetere, 2000; Hanayama et al., 2002). Changes in the properties of their surface membrane would improve the combination with a targeting approach such as immunotherapy.

Since the tumoricidal effect of HSV-tk/GCV treatment is conceivable via DNA damage, the loss of function of p53 can develop resistance to apoptosis. Li et al. reported, however, the HSV-tk/GCV cytotoxic response did not depend on the expression of a functional p53 (Li et al., 1999; Craperi et al., 1999). Similarly, we demonstrated an obvious tumoricidal effect without either p53 or p21 expression (Figs. 4 and 6). These results were consistent in all cell lines and suggested that p53-dependent cell cycle regulation or apoptosis induction may not correlate with the tumoricidal effect of HSV-tk/GCV treatment to oral SCC. Alternatively, HSV-tk/GCV treatment might have considerable potential to oral SCC that highly expresses mutated p53 *in vivo* (Naglar et al., 2002) and *in vitro* (Sakai and Tsuchida, 1992). A previous exogenous *p53* transduction study suggested co-expression of p53 did not enhance the cytotoxicity of HSV-tk/GCV treatment, although p53 was able to increase amount of apoptosis which was markedly less than the total cell death *in vitro* (Xie et al., 1999).

Dimerization between Bax and Bcl-2 is an important factor to direct either death promotion or death inhibition. Bax accumulation was reported after GCV exposure in glioma cell lines (Craperi et al., 1999). However, they could not rule out the possible occurrence of necrotic cell death. In contrast, over-expression of Bcl-2 inhibited HSV-tk/GCV-induced activation of caspase and apoptosis (Beltinger et al., 2000). However, no synchronous correlation could be found between the expression levels of Bcl-2 family and the sensitivity to the oral SCC cell lines.

No cleavage or activation of caspase-3 was detected in oral SCC cell lines. This suggested the existence of an alternative molecular pathway responsible for the tumoricidal effect of HSV-tk/GCV treatment to oral SCC cell lines even though the route of cell death is unknown.

One exception in the present study was an occasional positive TUNEL signal in SCCKN cells (Fig. 7). However, no other evidence of apoptosis was detected with other biological assays. Cautionary notes suggested that a positive TUNEL signal should not be considered as a specific marker of apoptosis because the assay would also suggest necrotic cell death (Charriaut-Marlangue and Ben-Ari, 1995; Grask-kraupp et al., 1995).

Apoptosis was originally described on the basis of the morphological features by electron microscopy (Kerr et al., 1972) even though some features may also be detectable by light microscopy. Apoptotic bodies including cellular remnants should be observed either in the intercellular space or within the cytoplasm of intact cells. A large number of treated cells showed disintegration of the cell structure involving irregular scattered heterochromatin, swelling of cytoplasm and intracellular organelles, and cytoplasmic membrane disruption in all cell lines. Furthermore, intact cells containing apoptotic bodies that deposit the fragmented components from neighboring dead cells were not found. Based on the annexin V/PI double staining at 72 h, the HSC-4 cell line should include the cells of early stage apoptosis of 19.1% (Fig. 5). However, we could not find firm evidence of apoptosis in semi- and ultra-thin section examinations. Consequently, apoptosis may not play a central role in the tumoricidal mechanism. The hypothesis that the bystander effect is mediated by phagocytosis is unacceptable for oral SCC cells *in vitro*.

A previous study using Fas ligand demonstrated an artificial deficiency of caspase-8 resulted

in a switch of cell death from apoptosis to necrosis in Jurket cell lines (Kawahara et al., 1998). This implies that caspase activation itself was dispensable for determining cell death. Kitanaka and Kuchino (1999) suggested the existence of caspase-independent programmed cell death with necrotic-like morphology, that can be activated either alone or in concert with the caspase-dependent apoptotic program. Differential sensitivity and the type of cell death induced by HSV-tk/GCV treatment appear to be dependent upon cell-type difference, in addition to the status of the apoptosis-related gene.

In summary, we demonstrated that oral SCC cell lines exhibited an obvious sensitivity to HSV-tk/GCV treatment suggesting the presence of a p53- and caspase-3-independent death signaling pathway. However, the mechanism and the molecular pathway responsible for HSV-tk/GCV-induced cell death are still unknown. A better understanding of the cell death mechanism is essential to establish an appropriate and effective modality in future studies.

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References

1 Beltinger C, Fulda S, Kammertoens T, Meyer E, Uckert W, Debatin KM. Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. Proc Natl Acad Sci U S A 1999;96:8699–8704.

2 Beltinger C, Fulda S, Kammertoens T, Uckert W, Debatin KM. Mitochondrial amplification of death signals determines thymidine kinase/ganciclovir-triggered activation of apoptosis. Cancer Res 2000; 60:3212–3217.

3 Bi WL, Parysek LM, Warnick R, Stambrook PJ. In vitro evidence that metabolic cooperation is responsible for the bystander effect observed with HSV tk retroviral gene therapy. Hum Gene Ther 1993;4: 725–731.

4 Borrelli E, Heyman R, Hsi M, Evans RM. Targeting of an inducible toxic phenotype in animal cells. Proc Natl Acad Sci U S A 1988;85:7572–7576.

5 Boucher PD, Ruch RJ, Shewach DS. Differential ganciclovir-mediated cytotoxicity and bystander killing in human colon carcinoma cell lines expressing herpes simplex virus thymidine kinase. Hum Gene Ther 1998;9:801–814.

6 Charriaut-Marlangue C, Ben-Ari Y. A cautionary note on the use of the TUNEL stain to determine apoptosis. Neuroreport 1995;7:61–64.

7 Chen CY, Chang YN, Ryan P, Linscott M, McGarrity GJ, Chiang YL. Effect of herpes simplex virus thymidine kinase expression levels on ganciclovir-mediated cytotoxicity and the "bystander effect". Hum Gene Ther 1995;6:1467–1476.

8 Colombo BM, Benedetti S, Ottolenghi S, Mora M, Pollo B, Poli G, et al. The "bystander effect": association of U-87 cell death with ganciclovir-mediated apoptosis of nearby cells and lack of effect in athymic mice. Hum Gene Ther 1995;6:763–772.

9 Craperi D, Vicat JM, Nissou MF, Mathieu J, Baudier J, Benabid AL, et al. Increased bax expression is associated with cell death induced by ganciclovir in a herpes thymidine kinase gene-expressing glioma cell line. Hum Gene Ther 1999;10:679–688.

10 Depraetere V. "Eat me" signals of apoptotic bodies. Nat Cell Biol 2000;2:E104.

11 Elion GB. Mechanism of action and selectivity of acyclovir. Am J Med 1982;73:7–13.

12 Freeman SM, Abboud CN, Whartenby KA, Packman CH, Koeplin DS, Moolten FL, et al. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res 1993;53:5274–5283.

13 Fukui T, Hayashi Y, Kagami H, Yamamoto N, Fukuhara H, Tohnai I, et al. Suicide gene therapy for human oral squamous cell carcinoma cell lines with adeno-associated virus vector. Oral Oncol 2001;37:211–215.

14 Glaser T, Castro MG, Lowenstein PR, Weller M. Death receptor-independent cytochrome c release

and caspase activation mediate thymidine kinase plus ganciclovir-mediated cytotoxicity in LN-18 and LN-229 human malignant glioma cells. Gene Ther 2001;8:469–476.
15 Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995;21:1465–1468.
16 Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. Nature 2002;417:182–187.
17 Kaneko Y, Tsukamoto A. Gene therapy of hepatoma: bystander effects and non-apoptotic cell death induced by thymidine kinase and ganciclovir. Cancer Lett 1995;96:105–110.
18 Katabi M, Yuan S, Chan H, Galipeau J, Batist G. The nonapoptotic pathway mediating thymidine kinase/ganciclovir toxicity is reduced by signal from adenovirus type 5 early region 4. Mol Ther 2002;5: 170–176.
19 Kawahara A, Ohsawa Y, Matsumura H, Uchiyama Y, Nagata S. Caspase-independent cell killing by Fas-associated protein with death domain. J Cell Biol 1998;143:1353–1360.
20 Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239–257.
21 Kitanaka C, Kuchino Y. Caspase-independent programmed cell death with necrotic morphology. Cell Death Differ 1999;6:508–515.
22 Lanuti M, Gao GP, Force SD, Chang MY, El Kouri C, Amin KM, et al. Evaluation of an E1E4-deleted adenovirus expressing the herpes simplex thymidine kinase suicide gene in cancer gene therapy. Hum Gene Ther 1999;10:463–475.
23 Li PX, Ngo D, Brade AM, Klamut HJ. Differential chemosensitivity of breast cancer cells to ganciclovir treatment following adenovirus-mediated herpes simplex virus thymidine kinase gene transfer. Cancer Gene Ther 1999;6:179–190.
24 Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl Iodine-124 labelled Annexin-V as a potential radiotracer to study apoptosis using positron emission tomography. J Exp Med 1995;182: 1545–1556.
25 Moolten FL, Wells JM, Heyman RA, Evans RM. Lymphoma regression induced by ganciclovir in mice bearing a herpes thymidine kinase transgene. Hum Gene Ther 1990;1:125–134.
26 Nagler RM, Kerner H, Laufer D, Ben-Eliezer S, Minkov I, Ben-Itzhak O. Squamous cell carcinoma of the tongue: the prevalence and prognostic roles of p53, Bcl-2, c-erbB-2 and apoptotic rate as related to clinical and pathological characteristics in a retrospective study. Cancer Lett 2002;186:137–150.
27 O'Malley BW, Cope KA, Chen SH, Li D, Schwarta MR, Woo SL. Combination gene therapy for oral cancer in a murine model. Cancer Res 1996;56: 1737–1741.
28 Rivas C, Miller AR, Collado M, Lam EW, Apperley JF, Melo JV. BCR-ABL-expressing cells transduced with the HSV-tk gene die by apoptosis upon treatment with ganciclovir. Mol Ther 2001;3:642–652.
29 Sakai E, Tsuchida N. Most human squamous cell carcinomas in the oral cavity contain mutated p53 tumor-suppressor genes. Oncogene 1992;7:927–933.
30 Samejima Y, Meruelo D. "Bystander killing" induces apoptosis and is inhibited by forskolin. Gene Ther 1995;2:50–58.
31 Vile RG, Castleden S, Marshall J, Camplejohn R, Upton C, Chong H. Generation of an anti-tumour immune response in a non-immunogenic tumour: HSVtk killing in vivo stimulates a mononuclear cell infiltrate and a Th1-like profile of intratumoural cytokine expression. Int J Cancer 1997;71:267–274.
32. Wei SJ, Chao Y, Hung YM, Lin WC, Yang DM, Shih YL, et al. S- and G2-phase cell cycle arrests and apoptosis induced by ganciclovir in murine melanoma cells transduced with herpes simplex virus thymidine kinase. Exp Cell Res 1998;241:66–75.
33 Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980;284:555–556.
34 Xie Y, Gilbert JD, Kim JH, Freytag SO. Efficacy of adenovirus-mediated CD/5-FC and HSV-1 thymidine kinase/ganciclovir suicide gene therapies concomitant with p53 gene therapy. Clin Cancer Res 1999;5:4224–4232.

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