Caspase-3-dependent apoptosis in cardiac myxoma: not associated with human papillomavirus or Epstein–Barr virus

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Cardiac myxoma is the most common tumor of the heart, has a variable clinical presentation and immunohistochemical profile. Viral infections, such as herpes simplex virus, human papillomavirus (HPV), and Epstein–Barr virus (EBV), may play an important role in the causes of cardiac myxoma. This investigation will demonstrate caspase-3-dependent apoptosis in cardiac myxoma without HPV or EBV infection. This study included 15 patients with cardiac myxoma, who were treated with surgical excision of the lesion. Data were collected on detailed clinical parameters. Terminal deoxynucleotidyl transferase nick-end labeling assay, electrophoresis, and caspase-3 immunohistochemical studies were performed to characterize apoptosis. Genechip containing 39 subtypes was used to elucidate HPV; and polymerase chain reaction to detect LMP-1 gene of EBV. The patient population comprised of eight (53%) women and seven (47%) men. The mean age of patient participants was 45 years, with an age range of 30–70 years. All patient cases were sporadic myxomas rather than familial myxomas. The patient presentations included dyspnea (53%), asymptomatic (27%), stroke (7%), chest pain (7%), and fever (7%). All lesions were located in the left atrium. The individual patient cases of myxoma did not differ in location or clinical event in terms of pathological scores, such as vascular proliferation, inflammation, cellularity, hyaline, calcification, or thrombosis. Cardiac myxoma is characterized by apoptosis through caspase-dependent pathway. HPV or EBV was not detected in any of the study patient samples. In conclusion, no viral genomes of HPV or EBV were detected in these 15 patients. This study demonstrates that caspase-3-dependent apoptosis in cardiac myxoma is not dependent on concurrence of previous HPV and/or EBV infection.
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Viruses play a role in causing cardiovascular disease. The viruses and virus classes that most frequent are the common cause of cardiomyopathy are Parvo B19, enteroviruses, adenoviruses, and cytomegalovirus (CMV). Epstein–Barr virus (EBV) and influenza virus cause cardiomyopathy, but less frequently[1,2] than previously mentioned viruses. Recent treatment advances have witnessed the long-term survival of patients with HIV/AIDS, allowing for larger numbers of cardiac manifestations of HIV disease to be observed. Cardiac manifestations of long-term HIV-disease survival include pericardial effusion, myocarditis, dilated cardiomyopathy, endocarditis, pulmonary hypertension, malignant neoplasms, and drug-related cardiotoxicity.[3] The relationship between viruses and

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cardiac tumors, such as cardiac lymphoma and papillary fibroelastoma, has been established.[4–6] Studies have proven that herpes simplex virus (HSV) is also associated with cardiac myxoma.[7]

Myxoma is the most common benign neoplasm of the heart.[8–15] Cardiac myxoma is associated with many mucopolysaccharidic matrices, including glycosaminoglycans[14,15] and proteoglycans. These glycoproteins may serve as receptors for the entry of HSV-1,[16,17] human papillomavirus (HPV),[18] and EBV.[19] In addition, the presence of the tumorigenic viruses, HSV and HPV, has been implicated to potentiate neoplastic conditions in mucin-enriched cornea and in conjunctiva.[20–23] HPV may play a major role in the development of rapidly progressive, multifocal transitional cell carcinoma in immunosuppressed patients. E6 and E7 genes of the oncogenic HPV-16 can be transfected into primary human myocardial fibroblasts, immortalizing them. Telomerase activity is detected in these transfected cell lines.[24] In addition, EBV has been implicated in the pathogenesis of Burkitt's lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma, nasopharyngeal carcinoma, and lymphomas, as well as leiomyosarcomas arising in immunocompromised individuals.[25] All of the above characteristics imply that HPV and EBV may play a role in cardiac myxoma.

We have previously reported apoptosis in cardiac myxoma; however, the exact relationship between apoptosis and cardiac myxomas is poorly understood. This investigation attempts to clarify the mechanisms leading to the development of apoptosis and its relationship to HPV and EBV.

Materials and methods

This study, conducted between June 2001 and December 2003, included 15 consecutive patients with cardiac myxoma who were treated by surgical excision at the Chung Gang Memorial Hospital, Taiwan. At that time, we obtained detailed clinical parameters. Information on age, sex, presenting symptoms, echocardiographic characteristics, and surgical procedures was obtained from patient medical records. Follow-up data were gathered from clinical records and from standardized telephone interviews. Tumor size and morphologic features for each patient were retrieved from surgical pathology reports. All sections were stained with hematoxylin–eosin and examined for vascular proliferation, inflammation, cellularity, hyaline, calcification, thrombosis, fibrosis, and Gamna–Gandy bodies. The clinical and pathologic features of the cardiac myxomas were assessed statistically to identify morphological features related to embolism, atrial fibrillation, and patient age on diagnosis. In addition, echocardiographic features were compared using the pathologic features.

Our institutional review board approved the study and all patients signed an informed consent form prior to enrollment into the study.

Genomic DNA Extraction and Electrophoresis

Genomic DNA was extracted using proteinase K and the phenol chloroform extraction method.[13,26,27] Samples were further purified through the use of a DNAeasy kit (Qiangen Inc., Venlo, The Netherlands). Finally, 1 μg of DNA solution was eluted and 1 μg of the aliquot was used for polymerase chain reaction (PCR) amplification. For electrophoresis, 1 μg of DNA was labeled with *Escherichia* Coli DNA polymerase and resolved with 1.5% agarose gel. The gel was dried and exposed for autoradiography.[13]

HPV Genotyping by Genechip

The SPF1/GP6 + consensus primers were used to amplify a fragment of approximately 184 bp in L1 open-reading frame first. Next, each PCR experiment was performed with several positive and negative controls.[26] The quality of isolated DNA was checked with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR. The resulting amplimers of 15 μg were then hybridized with an HPV genechip (Easy-chip HPV Genotyping Array, King Car, Taiwan). In all, 39 types of HPV ligonucleotide probes of 20- and 30-mer, with an approximately 100–200 poly-T tail, were immobilized on a nylon membrane.[26]

EBV Genotyping by PCR

For PCR amplification, oligonucleotide primers for detecting LMP-1 gene (sense BN1 [5′-AGC GAC TCT GCT GGA AAT GAT-3′] or antisense BN2 [5′-TGA TTA GCT AAG GCA TTC CCA-3′]) were used to examine the presence of extracted DNA.[27] The prepared sample was amplified for 35 cycles using the following procedures: denaturation at 94°C for 40 s, annealing at 50°C for 1 min, and extension at 72°C for 90 s in a programmable thermal controller (PTC-100, MJ Research Co.) without the overlay of mineral oil. Amplification of a genomic region in the hemoglobin gene (HEMHC-1 [5′-CGT CTC CTT TCC TCC GGA-3′] or HEMHC-2 [5′-CAC AGT GAC CTT CCC ATC-3′] served as a marker for the presence of intact genomic DNA. Negative control samples containing water were always processed in a manner parallel to the patient samples. DNA from the B95.8 cell line was used as the EBV-positive control sample.[27]

TUNEL Assay for Apoptosis

Samples from patients were assessed for apoptosis using the terminal deoxynucleotidyl transferase

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nick-end labeling (TUNEL) assay.[13] In order to detect DNA fragmentation *in situ*, the TUNEL assay was performed on deparaffinized 5 μm-thick myxoma sections according to the guidelines from the manufacturer (DNA Fragmentation Detection Kit; Oncogene Research). The DNAase I-treated section displayed positive control for TUNEL staining for apoptotic nuclei.

Immunohistochemical Stain for Caspase-3

We used the caspase-3 (H-277; Santa Cruz Biotechnology, Inc.) assay per manufacturer protocol.

The fraction of TUNEL and caspase-3-positive cells determined per 100 cardiac myxoma cells as follows: none (0) 1–20% (+), 21–40% (+ +), and 41–90% (+ + +).[13] The Student's *t*-test was utilized to make statistical comparison between the proportion groups.

Results

Clinical Findings

The patient population comprised eight (54%) women and seven (47%) men, with a mean age of 45 years, ranging in age from 30 to 70 years. All patients were cases of sporadic myxomas rather than familial myxoma. The breakdown for initial patient presentations was dyspnea (53%), asymptomatic (27%), stroke (7%), chest pain (7%), and fever (7%). For all patients participating in the study, cardiac myxoma tumors were confined to the left atrium. Tumors ranged in weight from 5 to 54 g (mean 28.6). No patients were noted to be experiencing atrial fibrillation prior to or during the study. All chest radiographs displayed nonspecific changes. All patients were mildly anemic, with average hemoglobin 11.3 mg/dl, most with patients experiencing mild mitral valve and tricuspid valve regurgitation. There were no perioperative mortalities or embolizations during the study period. At the time of this writing, all of the patients were alive. The mean time from patient treatment to the time of the authoring of this article was 10 months, with a range of 1–4.5 years. There were no patients with metastases or a recurrence of disease. Preoperative diagnosis was made by the use of transthoracic echocardiography.

Pathologic Findings

Microscopic examination revealed high levels of vascular proliferation (74%), hyaline formation (74%), congestive areas (67%), inflammation (54%), and multiple giant cells (47%) in the sample patients. On occasion, we noted hemorrhagic foci (14%) and calcification (14%). There were no cases of cardiac lithomyxoma in this study.

Expression of Apoptosis in Cardiac Myxoma

All cardiac myxomas patients had apoptosis-positive tissue samples (Figure 1), according to the following subgroups: 22% in the 11–20% apoptosis group, 22% in the 21–60% apoptosis group, and 66% in the >60% apoptosis group. The occurrence of apoptosis did not correlate with all clinical disease parameters, such as stroke, recurrence, or unusual sites other than the left atrium.

DNA Ladders Detected by Gel Electrophoresis

Nucleosomal fragmentation was studied using the end-labeling method as per manufacturer protocols. Following an autoradiogram-exposure time of 36 h, myxomas revealed DNA ladders from agarose gel electrophoresis (data not shown). Conversely, no DNA laddering was noted from normal heart tissues of the same time period.
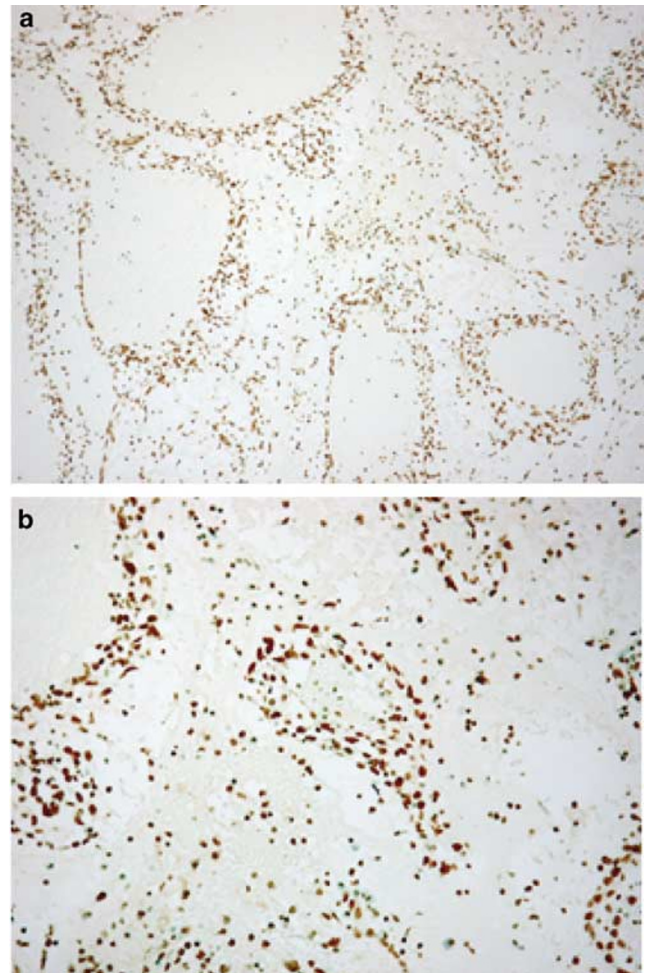

Figure 1 The pictures display typical apoptotic changes in cardiac myxoma. ((**a**) negative control; (**b**) positive apoptosis stain, arrows) (× 200).

Expression of Caspase-3 in Cardiac Myxomas

All immunochemical studies of caspase-3 were positive (Figure 2) and compatible with the apoptosis according to the following subgroups: 14% in the 11–20% apoptosis group, 20% in the 21–60% apoptosis group, and 67% in the >60% apoptosis group. This suggests that the caspase-3 pathway is activated in cardiac myxoma.

Negative Detection of HPV or EBV in Cardiac Myxoma

Detection limit of this assay was determined by serial dilution of plasmid DNA containing the specific segment of L1 open-reading frame. In the process, we used 39 HPV types and cell lines containing known HPV genotype, such as HeLa and CaSki cells, including between 10 and 100 copies per sample. None of the 39 (0%) HPV-type DNA sequences were detected on the genechip, and none of the EBV DNA sequences by PCR.
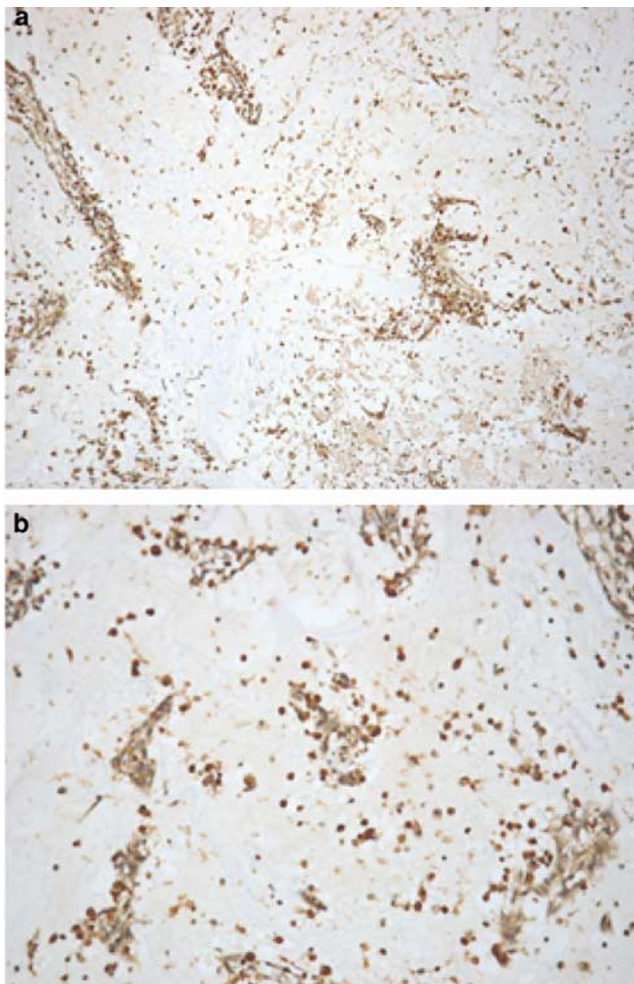

Figure 2 Representative pictures showing typical caspase-3 staining in cardiac myxoma ((**a**) negative control; (**b**) positive apoptosis stain, arrows) (×200).

Discussion

Myxoma is a benign neoplasm among cardiac tumors.[8–15] The morphologic studies, such as those on vascular proliferation, hyaline formation, congestive areas, inflammation, hemorrhagic foci, calcification, and multiple giant cells, were not statistically significant for clinical presentations as in previous reports.[8–12] Moreover, the findings of apoptotic changes in cardiac myxoma were compatible with previous studies.[13,28]

Apoptosis, commonly referred to as programmed cell death, involves the continuous destruction of nonfunctional cells.[29] Several pathways can be involved in programmed cell death. However, the molecular mechanisms that govern these processes remain unclear. Although apoptosis is involved in myxomas,[13,28,30] exactly how apoptosis and cardiac myxomas are related is poorly understood. Our first step was to provide clear evidence for a caspase-3-dependent pathway of apoptosis in cardiac myxoma by immunochemical studies. Secondly, this study attempts to clarify the relationship between cardiac myxoma, apoptosis, and HPV/EBV.[31]

One major pathway of apoptosis is in response to viral infection. Viruses have therefore evolved multiple distinct mechanisms for modulating host cell apoptosis. Previous studies have examined the consequences of HSV infection, including the induction of apoptosis.[31] Caspase-dependent apoptosis is involved in HSV-associated injury,[32] in adrenal cells, neuronal cells, corneal cells, hepatic cells, and dendritic cells as well.[33–37] Recently, the association between cardiac myxoma and HSV-1 has been determined.[7] Thus, we assume that HSV-1 might induce apoptosis in myxoma. However, our findings do not support the hypothesis that apoptotic myxoma cells are related to HPV or EBV; there are even some similarities between HSV and HPV.[16–20,21–23]

This investigation has several limitations. First, this retrospective study had a relatively small sample group. Second, the definition of apoptosis, as opposed to necrosis, remains controversial. We performed DNA fragmentation assays to verify the presence of apoptosis. While necrosis is an unregulated process causing cell demise, apoptosis is ordered and regulated. Third, the quality of DNA extracted from the samples might have been inadequate, even though we checked each sample first by the housekeeping gene, GADPH.

Conclusions

Results of this study demonstrate that caspase-3-dependent apoptosis has a novel pathological pattern in cardiac myxomas. Although the apoptosis in cardiac myxoma may not require HPV or EBV, this finding has potential significance for therapeutic treatment based on the administration of these genes

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for tumor-suppressive and apoptosis-inducing activity.[38,39]

Acknowledgements

References

1 Maisch B, Ristic AD, Portig I, *et al.* Human viral cardiomyopathy. Front Biosci 2003;8(Suppl):39–67.

2 Pauschinger M, Chandrasekharan K, Noutsias M, *et al.* Viral heart disease: molecular diagnosis, clinical prognosis, and treatment strategies. Med Microbiol Immunol 2004;193:65–69.

3 Rerkpattanapipat P, Wongpraparut N, Jacobs LE, *et al.* Cardiac manifestations of acquired immunodeficiency syndrome. Arch Intern Med 2000;160:602–608.

4 Ito M, Nakagawa A, Tsuzuki T, *et al.* Primary cardiac lymphoma. No evidence for an etiologic association with Epstein–Barr virus. Arch Pathol Lab Med 1996; 120:555–559.

5 Maric I, Washington S, Schwartz A, *et al.* Human herpesvirus-8-positive body cavity-based lymphoma involving the atria of the heart: a case report. Cardiovasc Pathol 2002;11:244–247.

6 Grandmougin D, Fayad G, Moukassa D, *et al.* Cardiac valve papillary fibroelastomas: clinical, histological and immunohistochemical studies and a physiopathogenic hypothesis. J Heart Valve Dis 2000;9:832–841.

7 Li Y, Pan Z, Ji Y, *et al.* Herpes simplex virus type 1 infection associated with atrial myxoma. Am J Pathol 2003;163:2407–2412.

8 Burke PA, Virmani R. Cardiac myxoma: a clinicopathologic study. Am J Clin Pathol 1993;100:671–680.

9 Reynen K. Cardiac myxomas. N Engl J Med 1995; 333:1610–1617.

10 Pucci A, Gagliardotto P, Zanini C, *et al.* Histopathologic and clinical characterization of cardiac myxoma: review of 53 cases from a single institution. Am Heart J 2000;240:134–138.

11 Pinede L, Duhaut P, Loire R. Clinical presentation of left atrial cardiac myxoma. A series of 112 consecutive cases. Medicine 2001;80:159–172.

12 Acebo E, Val-Bernal JF, Gomez-Roman JJ, *et al.* Clinicopathologic study and DNA analysis of 37 cardiac myxomas: a 28-year experience. Chest 2003; 123:1379–1385.

13 Chu PH, Jung SM, Wu HH, *et al.* Apoptosis in primary cardiac tumor. Int J Clin Pract 2004;58:564–567.

14 Chu PH, Jung SM, Yeh TS, *et al.* Mucin genes expression in cardiac myxoma. Int J Clin Pract 2004; 58:306–309.

15 Chu PH, Jung SM, Yeh TS, *et al.* MUC2 and MUC5AC expressions in cardiac myxoma. Virchows Archiv 2004, Nov 26; [Epub ahead of print] DOI: 10.1007/s00428-1147-5.

16 Detorakis ET, Sourvinos G, Spandidos DA. Detection of herpes simplex virus and human papilloma virus in ophthalmic pterygium. Cornea 2001;20:164–167.

17 McDonnell JM, McDonnell PJ, Sun YY. Human papillomavirus DNA in tissues and ocular surface swabs of patients with conjunctival epithelial neoplasia. Invest Ophthalmol Vis Sci 1992;23:184–189.

18 McDonnell JM, Mayr AJ, Martin WJ. DNA of human papillomavirus type 16 in dysplastic and malignant lesions of the conjunctiva and cornea. N Engl J Med 1989;320:1442–1446.

19 Guerreiro-Cacais AO, Li L, Donati D, *et al.* Capacity of Epstein–Barr virus to infect monocytes and inhibit their development into dendritic cells is affected by the cell type supporting virus replication. J Gen Virol 2004;85:2767–2778.

20 Eng HL, Lin TM, Chen SY, *et al.* Failure to detect human papillomavirus DNA in malignant epithelial neoplasms of conjunctiva by polymerase chain reaction. Am J Clin Pathol 2002;117:429–436.

21 Yanuck MD, Kaufman RH, Woods KV, *et al.* Cervical carcinoma metastatic to the skull, heart, and lungs: analysis for human papillomavirus DNA. Gynecol Oncol 1991;42:94–97.

22 Harms W, Rothamel T, Miller K, *et al.* Characterization of human myocardial fibroblasts immortalized by HPV16 E6–E7 genes. Exp Cell Res 2001;268:252–261.

23 Eiben GL, Velders MP, Schreiber H, *et al.* Establishment of an HLA-A*0201 human papillomavirus type 16 tumor model to determine the efficacy of vaccination strategies in HLA-A*0201 transgenic mice. Cancer Res 2002;62:5792–5799.

24 Takeda Y, Mori T, Imabayashi H, *et al.* Can the life span of human marrow stromal cells be prolonged by bmi-1, E6, E7, and/or telomerase without affecting cardiomyogenic differentiation? J Gene Med 2004;6:833–845.

25 Thompson MP, Kurzrock R. Epstein–Barr virus and cancer. Clin Cancer Res 2004;10:803–821.

26 Huang HJ, Huang SL, Lin CY, *et al.* Human papillomavirus genotyping by a polymerase chain reaction-based genechip method in cervical carcinoma treated with neoadjuvant chemotherapy plus radical surgery. Int J Gynecol Cancer 2004;14:639–649.

27 Tsang NM, Chang KP, Lin SY, *et al.* Detection of Epstein–Barr virus-derived latent membrane protein-1 gene in various head and neck cancers: is it specific for nasopharyngeal carcinoma? Laryngoscope 2003;113: 1050–1054.

28 Suzuki M, Hamada M, Hiwada K. Apoptosis in cardiac myxoma. Ann Intern Med 2000;132:681.

29 Kang PM, Yue P, Izumo S. New insights into the role of apoptosis in cardiovascular disease. Circ J 2002; 66:1–9.

30 Hofstra L, Dumont EA, Thimister PW, *et al. In vivo* detection of apoptosis in an intracardiac tumor. JAMA 2001;285:1841–1842.

31 Thomson BJ. Viruses and apoptosis. Int J Exp Pathol 2001;82:65–76.

32 Galvan V, Brandimarti R, Roizman B. Herpes simplex virus 1 blocks caspase-3-independent and caspase-dependent pathways to cell death. J Virol 1999;73: 3219–3226.

33 Aita K, Irie H, Koyama AH, *et al.* Acute adrenal infection by HSV-1: role of apoptosis in viral replication. Arch Virol 2001;146:2009–2020.

34 Perkins D, Gyure KA, Pereira EF, *et al.* Herpes simplex virus type 1-induced encephalitis has an apoptotic

component associated with activation of c-Jun *N*-terminal kinase. J Neurovirol 2003;9:101–111.

35 Miles D, Athmanathan S, Thakur A, *et al.* A novel apoptotic interaction between HSV-1 and human corneal epithelial cells. Curr Eye Res 2003;26:165–174.

36 Hashimoto K, Minagawa H, Yanagi Y. Caspase-dependent apoptosis in fulminant hepatic failure induced by herpes simplex virus in mice. J Hepatol 2003;39: 773–778.

37 Muller DB, Raftery MJ, Kather A, *et al.* Frontline: Induction of apoptosis and modulation of c-FLIPL and p53 in immature dendritic cells infected with herpes simplex virus. Eur J Immunol 2004;34:941–951.

38 Opalka B, Dickopp A, Kirch HC. Apoptotic genes in cancer therapy. Cells Tissues Organs 2002;172: 126–132.

39 Blattman JN, Greenberg PD. Cancer immunotherapy: a treatment for the masses. Science 2004;305:200–205.