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研究成果の刊行に関する一覧表
平成22年度 国立感染症研究所 感染病理部 片野晴隆

書籍

著者氏名	論文タイトル	書籍全体の編集者	書籍名	出版社名	出版地	出版年	ページ
片野晴隆、佐多徹太郎	Primary effusion lymphoma原発性滲出液リンパ腫	青笹克之	癌診療指針のための病理診断プラクティス リンパ球増殖疾患	中山書店	大阪	2010	197-203

研究成果の刊行に関する一覧表
平成22年度 国立感染症研究所 感染病理部 片野晴隆

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primary effusion lymphoma : PEL

腫瘍性疾患―非Hodgkinリンパ腫・B細胞性

原発性滲出液リンパ腫

疾患の概要

- PELは，胸水や腹水などの体腔液中に浮遊する形で増殖するB細胞性の液性リンパ腫であり，通常，腫瘍塊を形成しない．
- ヒトヘルペスウイルス8（human herpesvirus 8：HHV-8またはKaposi's sarcoma associated herpesvirus：KSHV）が腫瘍細胞核内に検出される．WHO分類の定義ではHHV-8陰性の症例はPELに含めない．したがって，**HHV-8の検出がPELの診断には必須である．**
- AIDSなどの免疫不全に関連して発症する例がほとんどを占める．
- 胸膜などの隣接する部位に固形腫瘍を形成することがある．また，まれに液性リンパ腫の形をとらず，HHV-8関連固形リンパ腫として現れることがあり，これはextracavity PEL（体腔外PEL）と呼ばれている．

染色体・遺伝子異常

- 報告されている染色体・遺伝子異常はない．

病態発生機構

- HHV-8による日和見腫瘍であり，すべてのリンパ腫細胞からHHV-8が検出される．HHV-8はEBVと同じγヘルペスウイルスに属し，Bリンパ球に潜伏感染するウイルスである 図1 ．

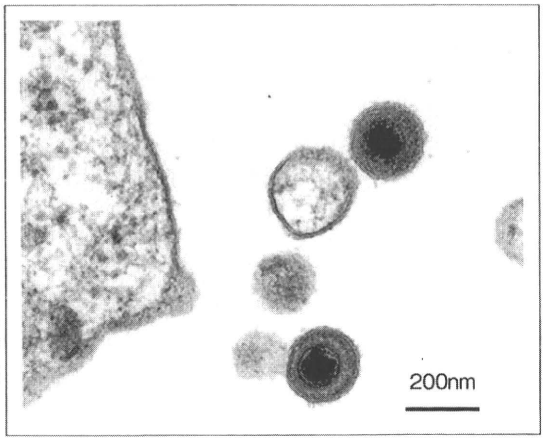


図1 HHV-8のウイルス粒子
フォルボルエステルで刺激したHHV-8持続感染細胞株BCBL-1に見られたウイルス粒子．他のHHVと近似し，約200nm程度の直径をもつ．なお，HHV-8は通常潜伏感染しているため，PELの臨床検体から電顕でウイルス粒子を見ることはできない．

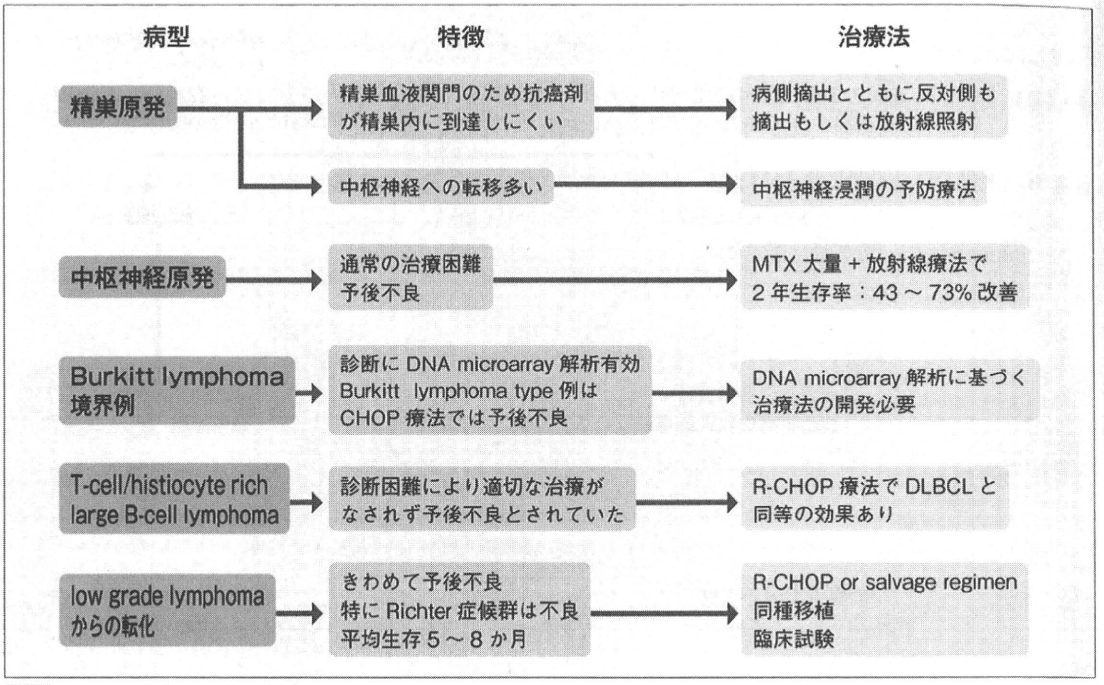


図13 DLBCL の特殊病型と治療法

トポシド，シタラビン，ブレオマイシン，ビンクリスチン）療法のランダム化比較試験において生存率に関して 4 者間に有意差がないことが 1993 年に報告され，以降，CHOP 療法が aggressive lymphoma の標準的治療法となった．1990 年代に B 細胞性リンパ腫に発現する CD20 に対するキメラ抗体薬剤リツキシマブが開発され，CHOP 療法とリツキシマブを併用する治療法が開発された．本治療法と CHOP 療法のランダム化比較試験にて DLBCL 例の生存率が 10～20%改善することが報告され，以後 DLBCL 治療の標準治療はリツキシマブ併用 CHOP 療法となった．

（青笹克之，水木満佐央）

表1 HHV-8の発癌に重要な遺伝子

発現時期	遺伝子名	PEL発症における働き
潜伏感染	LANA-1	HHV-8潜伏感染細胞で常に発現．p53, Rb1と結合し，アポトーシスの阻害，細胞周期の促進．HHV-8ゲノムの娘細胞への伝搬．潜伏感染の維持
	v-cyclin-D	cyclin D1のホモログ．Cdk6と結合し，細胞周期の促進
	vFILP	Fasを介したcaspase活性の抑制により，抗アポトーシス作用．NF-kBの活性化
	kaposin	形質転換に関与？
	LANA-2	PELに発現．IRFのホモログ
増殖感染	vIL-6	IL-6のホモログ．STAT3の恒常的活性化．VEGFの発現誘導．IFN-αの抗ウイルス作用を阻害
	vGPCR	GPCRのホモログ．細胞周期の促進
	vIRFs	IRFのホモログ．宿主サイトカインの制御．細胞周期の促進

- EBVと異なり，HHV-8はB細胞への単独での形質転換が証明されていないが，その遺伝子上にヒトサイトカイン，抗アポトーシス因子，細胞周期に関わる遺伝子のホモログが存在し，これらの発癌への関与が示唆されている 表1 ．
- PELやKaposi肉腫ではHHV-8は潜伏感染状態にあるが，ごく一部の細胞では増殖感染状態に移行し，増殖感染関連蛋白を発現する．特にHHV-8の潜伏感染蛋白であるlatency associated nuclear antigen 1（LANA-1）の働きは重要で，p53やRb1と結合し，細胞周期を促進する働きと，HHV-8 DNAを染色体に結合させ，細胞分裂時に宿主DNAとともにHHV-8 DNAを複製し，娘細胞にHHV-8を伝搬し感染を持続させる働きをもつ．
- 遺伝子発現プロファイルの解析から，PELはpost germinal center B-cellが由来と考えられ，plasma cellのマーカーであるCD138が陽性である．なお，EBVも多くのPEL症例で検出されるが，陰性の症例が存在することや，培養によりEBVのみが脱落する症例があることからEBVの感染はPELの病変形成に必須でないと考えられている．

臨床所見

既往歴等，好発年齢，性

- 若年～成年のHIV陽性男性同性愛者がほとんどを占める．約半数にはKaposi肉腫の合併がみられる．
- まれながら移植後のレシピエント，高齢者での発症がある．

初発症状

- 胸腔，腹腔，心嚢腔が好発部位であり，こうした体腔液中に滲出液（胸水，腹水，心嚢水）が貯留する．リンパ節腫脹を伴わないのが普通である．
- AIDS合併例ではKaposi肉腫の既往がある症例が多い．
- extracavity PELは消化管，皮膚，肺などが多い．

血液・血清所見

- 通常，白血化はない．
- 血清中の HHV-8 DNA 量が上昇する例が多く，血清中には HHV-8 に対する抗体が検出される．
- 血中の IL-6 量が増加するが，ウイルスがコードする viral IL-6（vIL-6）の量も増加する．

病理所見

病理標本の作製上の注意

- PEL は腫瘍塊として増殖しないため，病理標本の作製には工夫が必要である．胸水などのサンプルではリンパ腫細胞の smear やサイトスピン標本の作製が必要である．
- 細胞数が多ければ遠心して沈澱とし，パラフィン標本の作製も可能である．また，胸膜などの隣接する部位に形成された腫瘍塊や extracavity PEL では，病変部の固形腫瘍から通常の病理標本を作製する．

細胞診標本

- リンパ腫細胞の smear やサイトスピン標本の Giemsa 染色では，リンパ腫細胞は大型の immunoblastic または plasmablastic な細胞から，anaplastic large cell 様の形態をとり，多彩である 図2 ．
- 核は多形性に富み，明確な核小体が特徴的で，細胞質は比較的広く，好塩基性であり，plasma cell にみられる核周明庭を思わせる所見もみる．

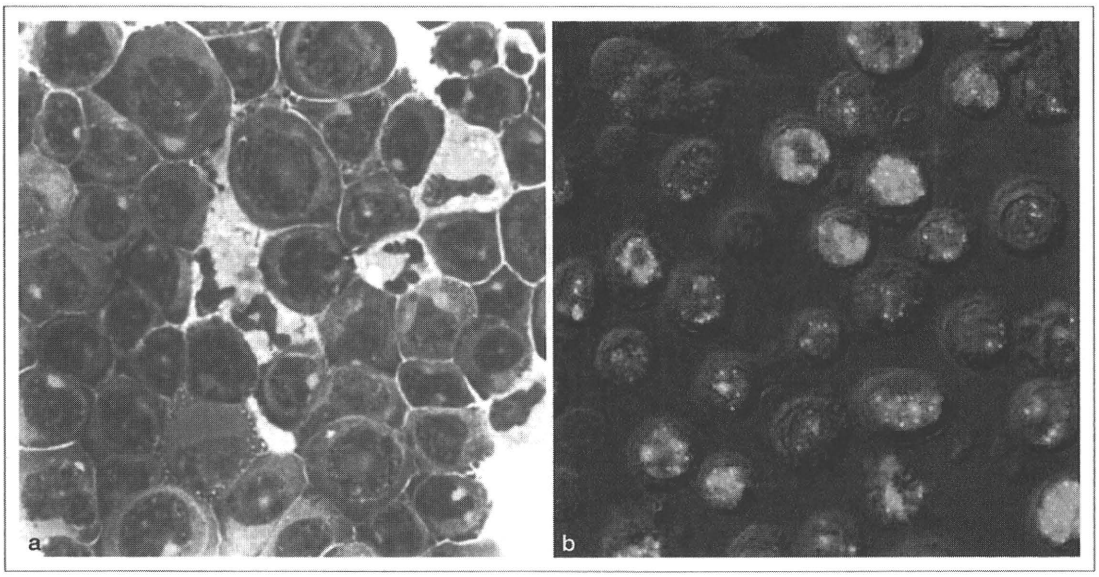


図2 PEL 症例の smear 標本
a：Giemsa 染色．大型の芽球様の細胞が見られる．核は多形性で，核小体が見られ，細胞質は広く，好塩基性に濃染する．核周明庭も見られ，plasma cell との類似を思わせる．
b：LANA-1 の蛍光免疫染色．ほとんどすべての細胞の核内に点状の陽性シグナルを認める．

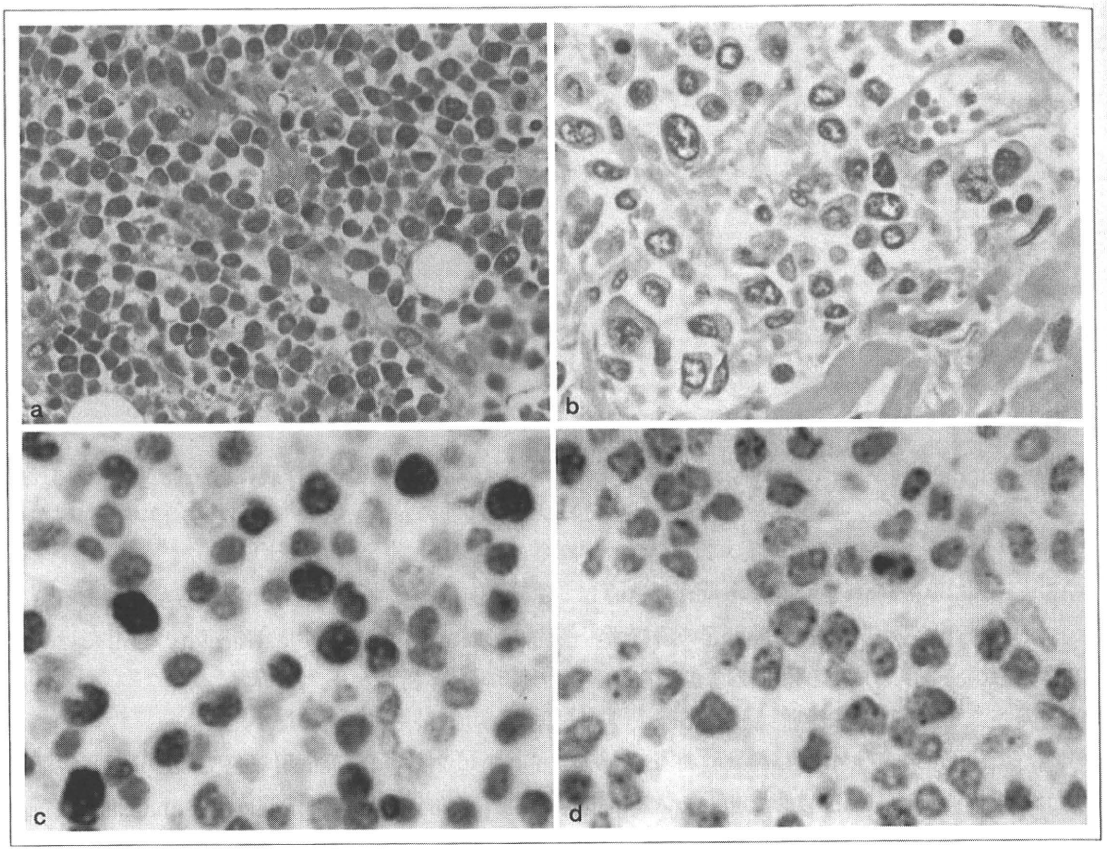


図3 extracavity PEL
a, b：HE 染色．大型の芽球様の細胞のびまん性浸潤を認める．核は偏在し，一部では車軸状である．細胞質は形質細胞に似る　c：EBV EBER の *in situ* hybridization　d：HHV-8 LANA-1 の免疫染色

■組織標本

- extracavity PEL のパラフィン標本では大型で多形性に富む plasmablast ないし anaplastic large cell 様の細胞がびまん性に浸潤し，周囲との境界は不明瞭である 図3．
- 核は明瞭な核小体を有し，クロマチンが車軸状に見えるものもある．好塩基性の豊富な細胞質には核周明庭がみられ，plasma cell 様に見えるものもある．

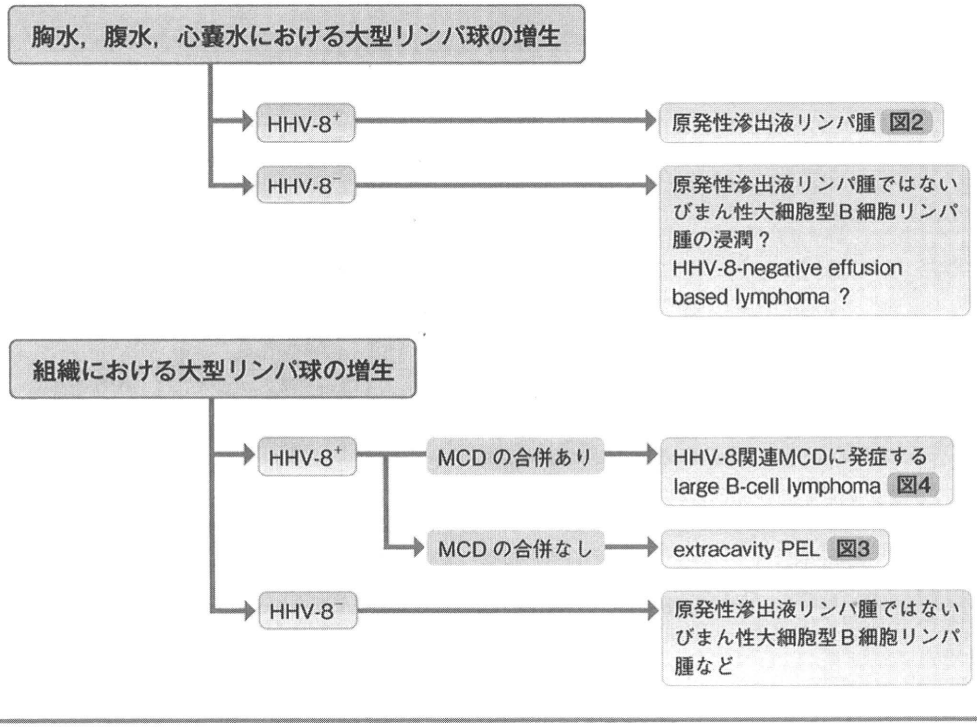
■免疫組織化学，*in situ* hybridization

- CD45（LCA）$^{+}$であるが，CD20，CD79a などの B 細胞マーカーはすべて脱落している．
- CD30^{+}，CD138^{+}になることが多い．
- HHV-8 の潜伏感染蛋白である LANA-1 がリンパ腫細胞には必ず陽性であり，これが唯一の PEL のマーカーといえる 図3d．
- EBV は EBV-encoded small RNAs（EBER）が *in situ* hybridization で検出されることが多いが，EBER^{+}例でも latent membrane protein 1（LMP-1）$^{-}$であることが多い．
- extracavity PEL も同様の表面マーカーを発現する．

その他

- real time PCR を用いるとリンパ腫細胞に HHV-8 DNA が約 50 copy/cell で検出される.
- リンパ腫細胞の遺伝子検査ではイムノグロブリンの遺伝子再構成が認められる.

鑑別診断



▶びまん性大細胞型 B 細胞リンパ腫(diffuse large B-cell lymphoma : DLBCL)

extracavity PEL は形態的に DLBCL と区別がつかない. HHV-8$^+$ であれば DLBCL と同様の形態を示していても extracavity PEL とする.

➡ extracavity PEL は plasmablastic または anaplastic large cell 様の形態をとること，B 細胞マーカーが欠如すること，CD30$^+$ である症例が多いことなどが参考となる.

▶ HHV-8 陰性液性リンパ腫 (HHV-8-negative effusion based lymphoma)

C 型（または B 型）肝炎患者の腹水中に PEL と形態的によく似た液性リンパ腫が発症することが報告されている.

➡これらは HHV-8$^-$ であり，PEL とは異なる疾患である.

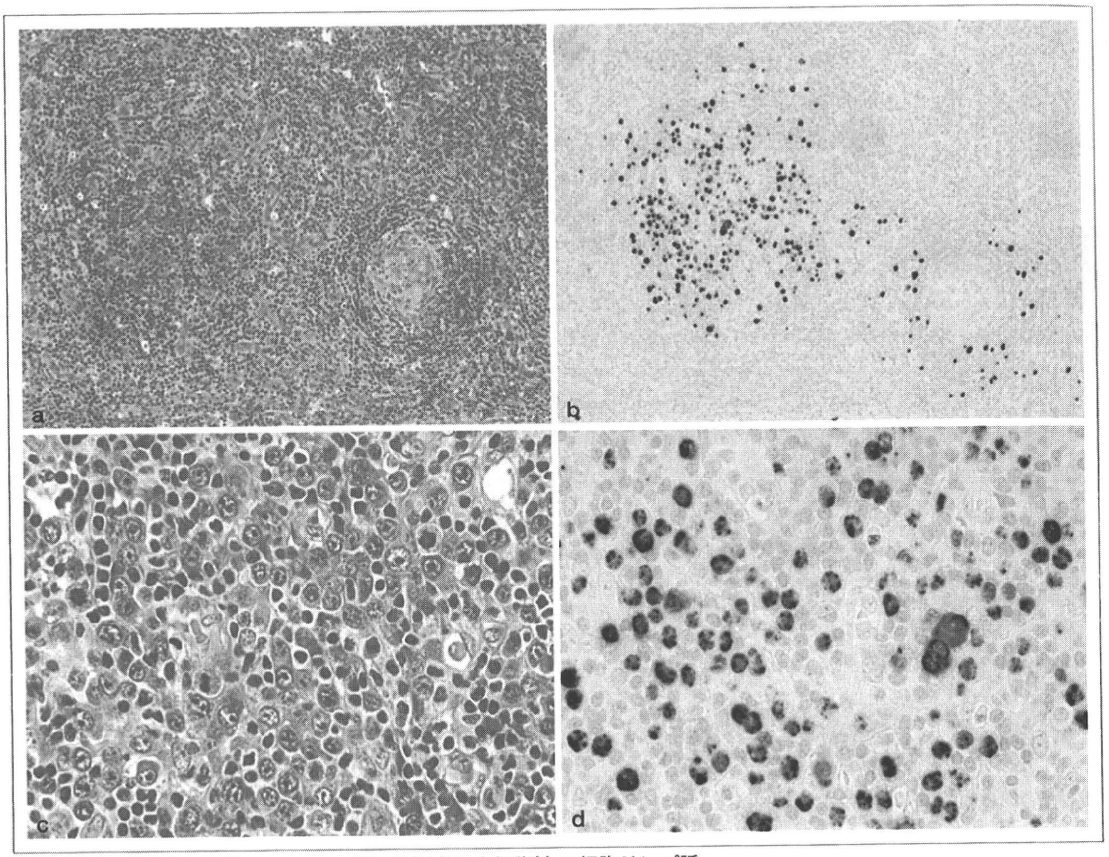


図4 HHV-8 関連多発性 Castleman 病に発症する大細胞性 B 細胞リンパ腫
a：HE 染色．右下には Castleman 病に典型的な硝子化した胚中心が見られ，暗殻は同心円状に拡大している．濾胞間には plasma cell の増生が見られる．
b：a の LANA-1 の免疫染色．リンパ濾胞暗殻と濾胞間組織に LANA-1$^+$細胞が見られる．
c：b の濾胞間組織における LANA-1$^+$細胞の HE 染色．plasma cell 様細胞が集簇している．
d：同部位の LANA-1 の免疫染色．plasma cell 様細胞に LANA-1$^+$で，この部位はいわゆる micro-lymphoma といえる（本文参照）．

▶膿胸関連リンパ腫（pyothorax-associated lymphoma：PAL）

PAL は結核性膿胸患者の胸膜に発症する EBV 関連の固形リンパ腫であり，HHV-8$^-$である．

➡ PEL は胸水中に液性リンパ腫として発症する点でも区別できる．

診断のポイント

- 本邦では HIV 陽性男性同性愛者に発症がほぼ限定されることから，既往歴の確認が重要である．
- 胸水，腹水などからリンパ腫細胞が検出され，通常，腫瘍塊を形成しない．
- HHV-8$^+$であることは PEL 診断の必須条件である．
- リンパ節腫大または脾腫がある場合は HHV-8 関連 multicentric Castleman 病（MCD）に発症する large B-cell lymphoma を疑う．

▶ HHV-8 関連多発性 Castleman 病に発症する大細胞性 B 細胞リンパ腫 (large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease) 図4

HHV-8 関連の多発性 Castleman 病（MCD）から発症するリンパ腫であり，PEL と同じ危険因子の患者（AIDS，男性同性愛者，Kaposi 肉腫合併）に発症する．形態的に plasma cell に似ることから HHV-8-positive plasmablastic lymphoma ともいわれる．

MCD を発生母地とすることから，リンパ節や脾臓に発症し，リンパ節腫脹や脾腫として現れる．組織学的には Castleman 病の病変部では胚中心の同心円状の硝子様物質の沈着と中心に向かって入り込む血管が特徴の，いわゆる onion skin appearance を示し，濾胞間領域には多くの plasma cell の増生が認められる．HHV-8 はマントル層および濾胞間の plasma cell の一部に陽性で，HHV-8$^+$細胞が小さな集塊を形成し，シート状に増生している像もみられる（micro-lymphoma と呼ばれる）．

免疫組織学的には HHV-8 LANA-1$^+$で，HHV-8 の溶解性感染関連蛋白である vIL-6 も多くの細胞で陽性である．

➡ cIgM$^+$，λ light chain$^+$である点は PEL とは異なる．Castleman 病の存在が PEL との鑑別には重要である．

治療

- 現在のところ，PEL に対する確立した治療法はない．無治療での生存期間の中央値は 2〜3 か月であり，一般に予後は悪い．
- CHOP 療法などの化学療法に加え，highly active anti-retroviral therapy（HAART）を併用することで治療成績の改善がみられる．HAART のみで PEL が消退した症例報告もみられ，HAART による免疫の再構築が PEL の治療にも有利に働くものと考えられる．
- PEL は CD20$^-$であるためにリツキシマブは使用されない．

（片野晴隆，佐多徹太郎）

Cancer Letters 300 (2011) 225–234

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Fucoxanthin and its deacetylated product, fucoxanthinol, induce apoptosis of primary effusion lymphomas

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ARTICLE INFO

Article history:
Received 5 September 2010
Received in revised form 15 October 2010
Accepted 20 October 2010

Keywords:
PEL
HHV-8
FX
FXOH
Proteasome

ABSTRACT

Primary effusion lymphoma (PEL) is a rare type of non-Hodgkin's lymphoma caused by human herpesvirus 8. Conventional chemotherapy has limited effect on PEL, and the prognosis is poor. Carotenoids are a family of natural pigments and have several biological functions. We evaluated the anti-PEL effects of carotenoid, fucoxanthin (FX) and its metabolite, fucoxanthinol (FXOH). Treatment of PEL cells with FX or FXOH induced cell cycle arrest during G_1 phase and caspase-dependent apoptosis. FX and FXOH treatment silenced NF-κB, AP-1 and Akt activation, in conjunction with down-regulation of anti-apoptotic proteins and cell cycle regulators. Importantly, proteasome degradation was responsible for the low levels of proteins after FXOH treatment. In animal studies, treatment with FX reduced the growth of PEL-cell tumors. The results provide the rationale for future clinical use of FX and FXOH for the treatment of PEL.

1. Introduction

Primary effusion lymphoma (PEL) is a rare, distinct subtype of non-Hodgkin's lymphoma that occurs exclusively in body cavities (pleura, peritoneum and pericardium) as serous lymphomatous effusion without tumor formation or organ infiltration [1,2]. The neoplastic cells have a B-cell genotype, but lack surface expression of some B-cell-associated antigens and surface immunoglobulin. The most unique characteristic of these cells is their consistent infection with human herpesvirus 8 (HHV-8), also called Kaposi sarcoma-associated herpesvirus [3]. PEL is found predominantly in patients with advanced acquired immune deficiency syndrome (AIDS) and those with preexisting Kaposi sarcoma [4]. The prognosis of patients with human immunodeficiency virus-related PEL remains poor, with reported median survival times of less than six months [5]. HHV-8 is a member of the γ-herpesvirus family and can infect various cell types, including B cells, which provide a reservoir for latent virus [6]. Encoded within its genome are a number of viral transforming genes and pirated cellular homologues that subvert cellular signaling pathways including those leading to the activation of nuclear factor-κB (NF-κB) and survival. In HHV-8-infected cells, the viral Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein (v-FLIP) interacts with tumor necrosis factor receptor-associated factor adaptor proteins, NF-κB-inducing kinase and IκB kinase (IKK) [7–10] with subsequent induction of NF-κB activity in latently infected lymphoma cells [11]. In addition to v-FLIP, HHV-8 proteins K1, K15, and the viral G protein-coupled receptor (v-GPCR), a homologue of the human interleukin (IL)-8 receptor, induce NF-κB activity, thereby supporting a central role for NF-κB signaling in HHV-8 pathogenesis [12].

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doi:10.1016/j.canlet.2010.10.016

This transcription factor exists as a dimmer of Rel family proteins that are maintained in an inactive form in the cytoplasm, and that, upon activation, translocate to the nucleus and upregulate the expression of various genes. Among the NF-κB target genes are inflammatory cytokines, anti-apoptotic proteins and cell cycle regulators that promote cellular longevity [13]. Previous studies reported that treatment with Bay 11-7082, an NF-κB inhibitor, selectively inhibited IκBα phosphorylation and constitutive NF-κB DNA-binding activity in KSHV-infected PEL cells [14]. PEL cells treated with Bay 11-7082 demonstrated down-regulation of the NF-κB inducible cytokine IL-6 and apoptosis.

Activator protein 1 (AP-1) is also known to regulate cell proliferation [15], and HHV-8 latency-associated nuclear antigen (LANA), v-FLIP and vGPCR activate AP-1 [16–20]. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is also reported to play a critical role in cell proliferation and survival in PEL [21,22]. vGPCR and HHV-8 K1 activate the PI3K/Akt pathway [23–25].

Fucoxanthin (FX), a xanthophyll derivative, is an orange-colored pigment present specifically in edible brown algae. FX has various beneficial activities such as anti-cancer activity against various types of cancer cells [26], anti-inflammatory activity [27] and anti-oxidant activity [28]. Orally administered FX is metabolized to fucoxanthinol (FXOH), which is further converted to amarouciaxanthin A. FXOH is a major gastrointestinal metabolite of dietary FX [29]. In search for a new treatment modality for PEL based on the concept of molecular targeting, we investigated the effects of FX and FXOH on PEL cells both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cells

PEL cell lines used in the experiments were BCBL-1 and TY-1 infected with HHV-8 [30,31]. Peripheral blood mononuclear cells (PBMC) from healthy volunteers were also analyzed. All samples were obtained after informed consent. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. HeLa cervical cancer cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin.

2.2. Reagents

FX was extracted from brown seaweed *Cladosiphon okamuranus Tokida* using acetone as solvent, and purified by column chromatography, liquid–liquid partition and recrystallization up to >95% purity. Further purification was performed by RP-HPLC up to >98% purity, for *in vitro* assay. FXOH was prepared by enzymatic hydrolysis of purified FX using porcine pancreatic lipase. For this purpose, 195 mg of FX, 2 g of sodium taurocholate and 2 g of porcine pancreatic lipase (Type II; Sigma–Aldrich, St. Louis, MO, USA) were dissolved in 30 ml of 0.1 M sodium phosphate buffer (pH 7.0). The reaction buffer was incubated at 37 °C for 3 h. FXOH was purified by ODS column chromatography, liquid–liquid partition and recrystallization. In the experiment, we prepared 142 mg of purified FXOH (>95% purity, 72% yield). Further purification was achieved by RP-HPLC up to >98% purity, for *in vitro* assay. The identity and purity of the products were confirmed by comparison with reference Fx (Wako Pure Chemical Industries, Osaka, Japan) and the data in literature.

Antibodies to cyclin D2, IκBα, JunB, JunD, NF-κB subunits p50, p65, c-Rel, p52 and RelB, and AP-1 subunits c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to Bax, Bcl-2, CDK4, CDK6, c-Myc, phospho-retinoblastoma protein (pRb) (Ser780) and actin were purchased from NeoMarkers (Fremont, CA, USA). Antibody to XIAP was obtained from Medical & Biological Laboratories (MBL; Nagoya, Japan). Antibodies to Hsp90 and β-catenin were purchased from BD Transduction Laboratories (San Jose, CA, USA). Antibodies to survivin, Bak, Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), PDK1, IKKα, IKKβ, phospho-IKKβ (Ser181), phospho-IκBα (Ser32 and Ser36), caspase-8, caspase-9, phospho-caspase-9 (Thr125), cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP) and Bcl-x_L were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody to IKKγ and the proteasome inhibitor *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) were obtained from Sigma–Aldrich.

2.3. Cell viability and assays of apoptosis

The effects of FX or FXOH on cell viability were assessed using the water-soluble tetrazolium (WST)-8 (Wako Pure Chemical Industries). Briefly, 1×10^5 cells/ml were incubated in a 96-well microculture plate in the absence or presence of various concentrations of FX or FXOH. After 24-h culture, WST-8 (5 μl) was added for the last 4 h of incubation and the absorbance at 450 nm was measured using an automated microplate reader. Mitochondrial dehydrogenase cleavage of WST-8 to formazan dye provided a measure of cell proliferation. Apoptotic events in cells were detected by staining with phycoerythrin-conjugated Apo2.7 monoclonal antibody (Beckman Coulter, Marseille, France) [32] and analyzed by flow cytometry (Epics XL, Beckman Coulter, Fullerton, CA, USA). For analysis of morphological changes in nuclei, cells were stained with 10 μg/ml Hoechst 33342 (Wako Pure Chemical Industries) and observed under Leica DMI6000 microscope (Leica Microsystems, Wetzlar, Germany).

2.4. Cell cycle analysis

Cell cycle analysis was performed with the CycleTEST PLUS DNA reagent kit (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Briefly, 1×10^6 cells were washed with a buffer solution containing sodium citrate, sucrose and DMSO, suspended in a solution containing RNase A, and then stained with 125 μg/ml propidium iodide for 10 min. Cell suspensions were analyzed on a Coulter EPICS XL using EXPO32 software. The population of

cells in each cell cycle phase was determined with Multi-Cycle software.

2.5. In vitro measurement of caspase activity

Caspase activity was measured with the Colorimetric caspase assay kits (MBL, Nagoya, Japan). Cell extracts were prepared using Cell Lysis buffer and assessed for caspase-3, -8 and -9 activities using colorimetric probes. Colorimetric caspase assay kits are based on detection of the chromophore *p*-nitroanilide after cleavage from caspase-specific-labeled substrates. Colorimetric readings were performed in an automated microplate reader at an optical density of 405 nm.

2.6. Western blot analysis

Cells were lysed in a buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to electrophoresis on SDS–polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with the specific antibodies. The bands were visualized by enhanced chemiluminescence (GE Healthcare Unlimited, Buckinghamshire, UK).

2.7. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cells were cultured and examined for inhibition of NF-κB and AP-1 after exposure to FXOH for 24 h. Nuclear proteins were extracted as described by Antalis and Goldbolt [33] with modifications, and NF-κB and AP-1 binding activities to NF-κB and AP-1 elements were examined by EMSA. Briefly, 5 μg of nuclear extracts were preincubated in a binding buffer containing 1 μg poly-deoxy-inosinic-deoxy-cytidylic acid (GE Healthcare Biosciences), followed by the addition of ^{32}P-labeled oligonucleotide probes containing NF-κB and AP-1 elements. The mixtures were incubated for 15 min at room temperature. The DNA protein complexes were separated on 4% polyacrylamide gels and visualized by autoradiography. The probes used were prepared by annealing the sense and antisense synthetic oligonucleotides; a typical NF-κB element from the IL-2 receptor α chain (IL-2Rα) gene (5′-gatcCGGCAGGGGAATCTCCCTCTC-3′) and an AP-1 element of the IL-8 gene (5′-gatcGTGATGACTCAGGTT-3′). The above underlined sequences represent the NF-κB and AP-1 binding sites.

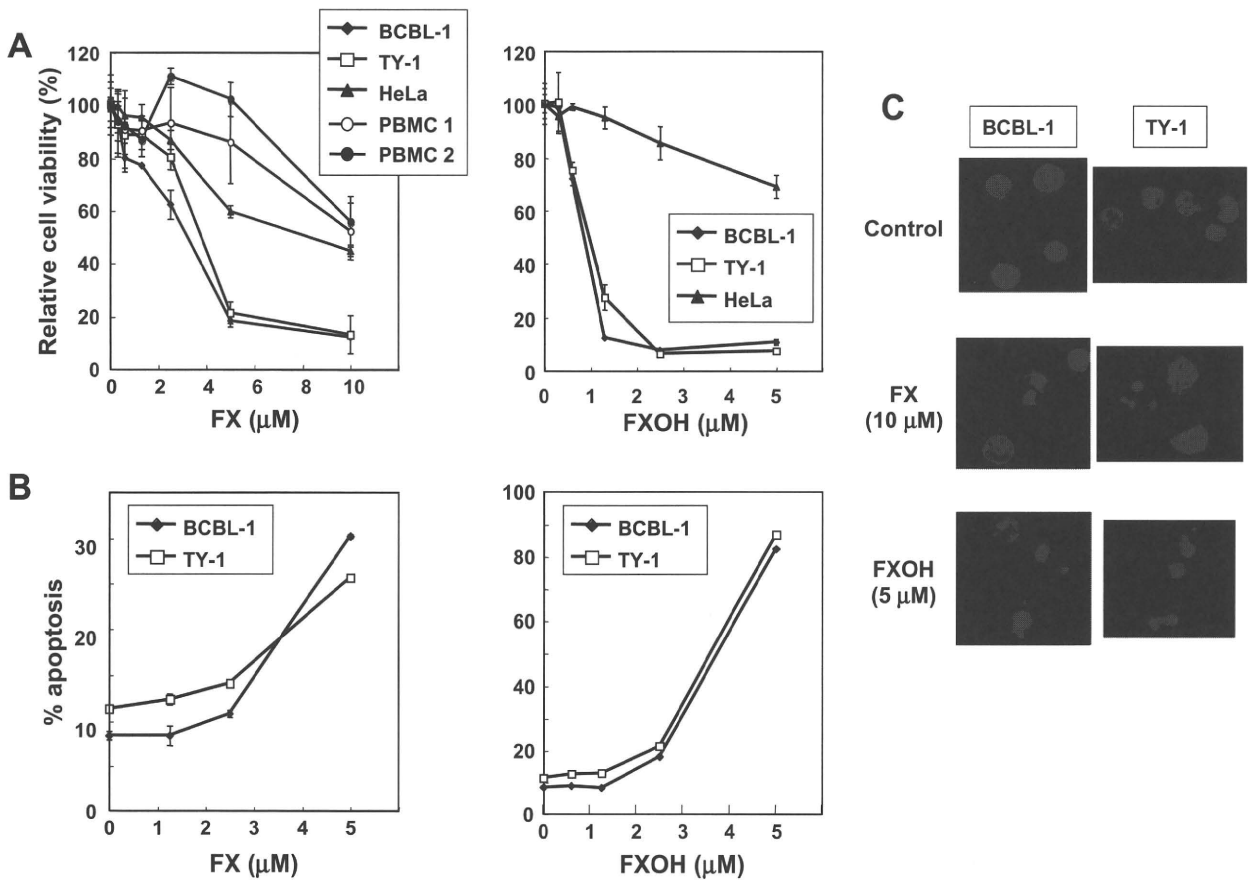

Fig. 1. FX and FXOH reduce cell viability and induce apoptosis of PEL cell lines. PEL cell lines, HeLa and PBMC from healthy controls were incubated in the presence of various concentrations of FX or FXOH for 24 h. (A) The viability of the cultured cells was determined by WST-8 assay. Relative viability of the cultured cells is presented as the mean ± SD determined for cells from triplicate cultures. (B) Cells were harvested, then stained with the Apo2.7 monoclonal antibody, and analyzed by flow cytometry. Data are mean ± SD percentages of apoptotic cells from triplicate cultures. (C) Hoechst 33342 staining. PEL cell lines were treated with FX (10 μM) or FXOH (5 μM) for 24 h and stained by Hoechst 33342. Original magnification, 630×.

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 5 μg of total RNA using a PrimeScript RT-PCR kit (Takara Bio, Otsu, Japan) with random primers. The primers for ORFK13 (v-FLIP), ORF72 (v-cyclin), ORF73 (LANA), ORF50 (Rta) and ORFK9 (v-IRF) were identical to those used in previous studies [34,35]. The PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

2.9. Analysis of in vivo therapeutic effect of FX

Five-week-old female C.B-17/Icr-scid [severe combined immunodeficiency (SCID)] mice obtained from CLEA Japan, Inc. (Tokyo) were maintained in containment level 2 cabinets and provided with autoclaved food and water *ad libitum*. Mice were engrafted with 5×10^6 BCBL-1 cells by intraperitoneal injection and then randomly placed into two cohorts of three mice each that received vehicle and FX, respectively. Treatment was initiated on the day after cell injection. FX was dissolved in soybean oil, and 150 mg/kg body weight of FX was administered by oral gavage every day for 56 days. All mice were sacrificed on day 56, and the tumors were dissected out and weighed. This experiment was performed according to the guidelines for Animal Experimentation of the University of the Ryukyus and approved by the Animal Care and Use Committee of the same University.

2.10. Statistical analysis

Data are expressed as mean ± SD. Body weight of mice and weight of tumors from FX-treated mice were compared with those of the vehicle-treated controls by the Mann–Whitney *U*-test. A *P* value below 0.05 was considered statistically significant.

3. Results

3.1. FX and FXOH reduce cell viability of PEL cell lines

We first examined the effects of FX and FXOH on the cell viability of PEL cell lines. Culture of cells in the presence of various concentrations of FX or FXOH for 24 h resulted in decreased cell viability in a dose-dependent manner in both BCBL-1 and TY-1 cells, as assessed by WST-8 assay

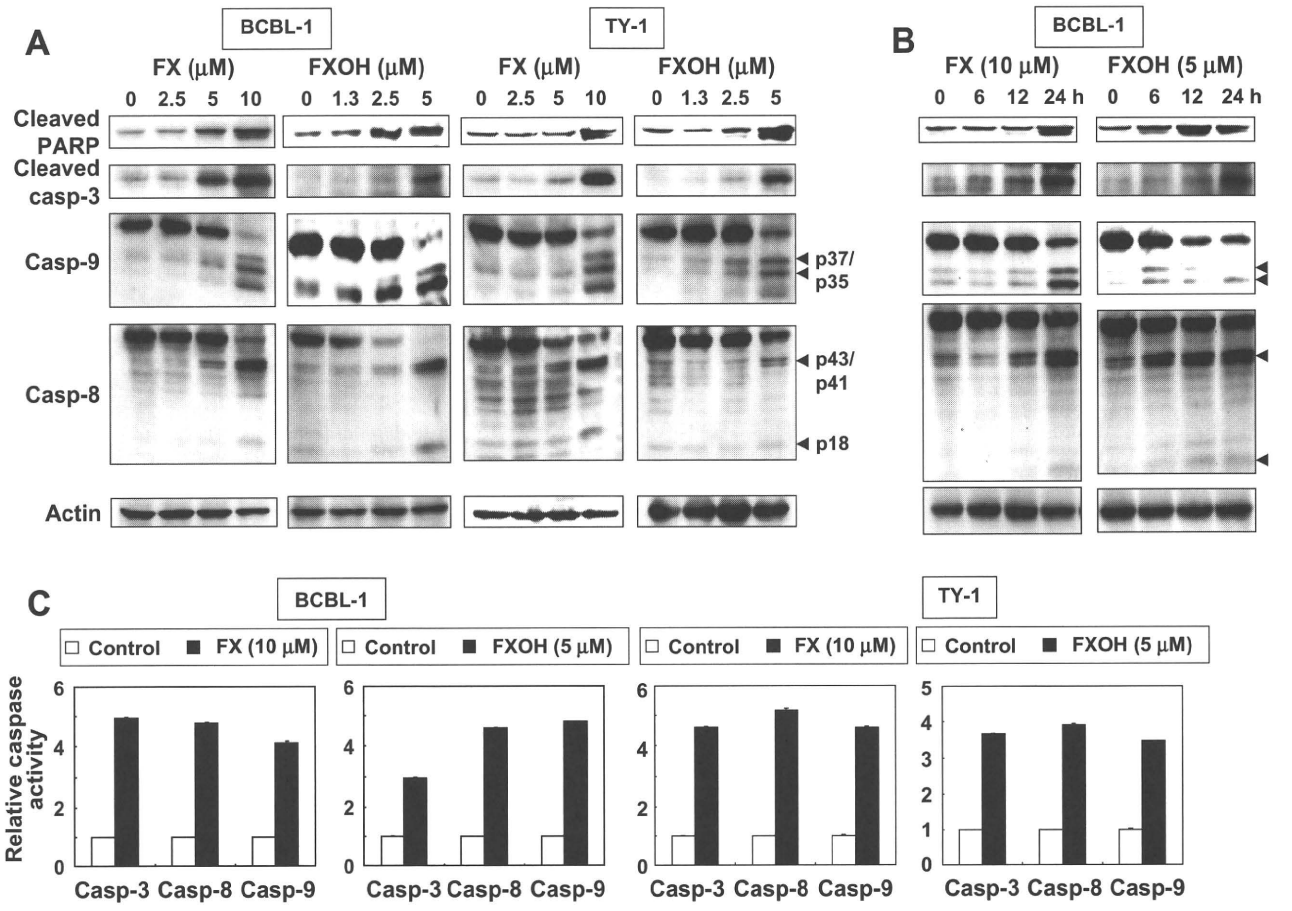

Fig. 2. FX- and FXOH-induced apoptosis is caspase-dependent. (A) BCBL-1 and TY-1 cells were incubated with the indicated concentrations of FX or FXOH for 24 h. (B) BCBL-1 cells were incubated with FX (10 μM) or FXOH (5 μM) for various time intervals. Cellular proteins were resolved by SDS/polyacrylamide gel electrophoresis, and caspase activity was detected by cleavage of PARP, caspase-3, caspase-9 and caspase-8 using immunoblot analysis. Arrowheads indicate the cleaved form of caspase-9 and caspase-8. Actin was used as a protein-loading control. (C) BCBL-1 and TY-1 cells were treated with or without FX (10 μM) or FXOH (5 μM). After 24 h, cell lysates were prepared and incubated with the labeled caspase substrates, and caspase activity was measured using an automated microplate reader. Caspase activity is expressed relative to untreated cells, which were assigned a value of 1. Values are mean ± SD ($n = 3$).

(Fig. 1A). FXOH-induced suppression of cell viability was more pronounced than that of FX with IC_{50} values of 1.1 and 2.4–3.3 μM, respectively. PBMC from healthy volunteers were resistant to FX (Fig. 1A). Interestingly, HeLa cervical cancer cells were less susceptible to FX and FXOH than PEL cell lines (Fig. 1A).

3.2. FX and FXOH induce apoptosis in PEL cell lines

We next examined whether induction of apoptosis accounted for the reduced cell viability observed in PEL cell lines. Cells were treated with various concentrations of FX and FXOH then probed with the Apo2.7 monoclonal antibody. FX and FXOH increased the proportion of apoptotic cells in PEL cell lines in a dose-dependent manner (Fig. 1B). Induction of apoptosis was further demonstrated by chromatin condensation and nuclear fragmentation noted with the use of Hoechst 33342 staining (Fig. 1C). Taken together, these results indicated that the inhibitory effects of FX and FXOH on the viability of PEL cell lines reflect their pro-apoptotic properties.

3.3. FX- and FXOH-induced apoptosis is caspase-dependent

We investigated whether the observed apoptosis was due to caspase activation. Cell extracts were obtained after various treatments and processed for immunoblot analysis. As shown in Fig. 2A and B, immunoblot analysis demonstrated the production of cleaved products of PARP, caspase-3, -8 and -9 by FX or FXOX in dose- and time-dependent manners. The immunoblotting allowed us to examine the processing of caspases, but did not indicate whether the cleavage products were enzymatically active. Therefore, caspase-3, -8 and -9 activities were determined by cleavage of caspase-specific-labeled substrates in colorimetric assays. FX and FXOH treatments resulted in activation of caspase-3, -8, and -9

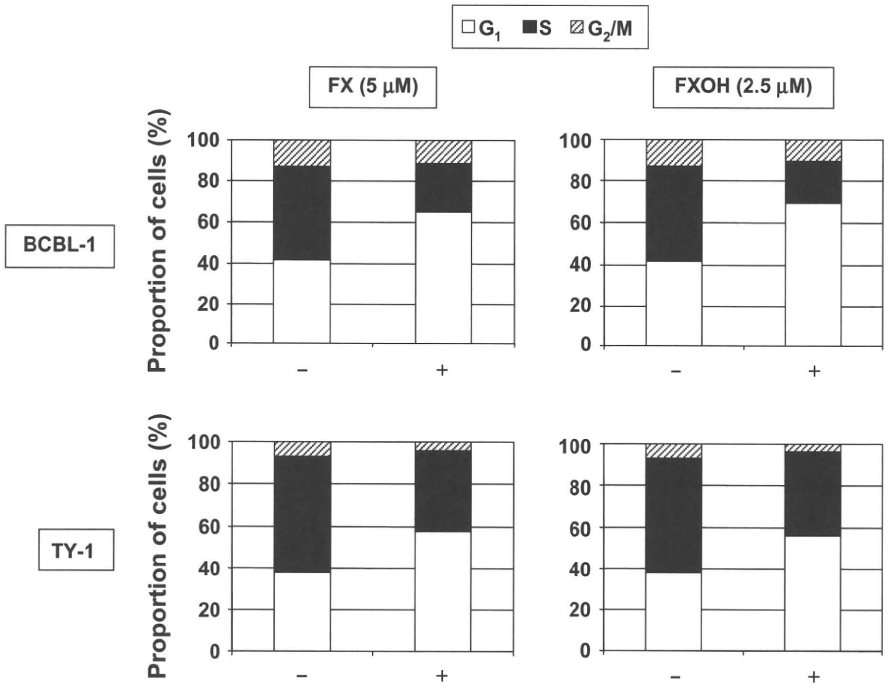

Fig. 3. FX and FXOH induce G_1 cell cycle arrest in PEL cell lines. BCBL-1 and TY-1 cells were incubated in the absence or presence of FX (5 μM) or FXOH (2.5 μM) for 24 h. Then, the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry.

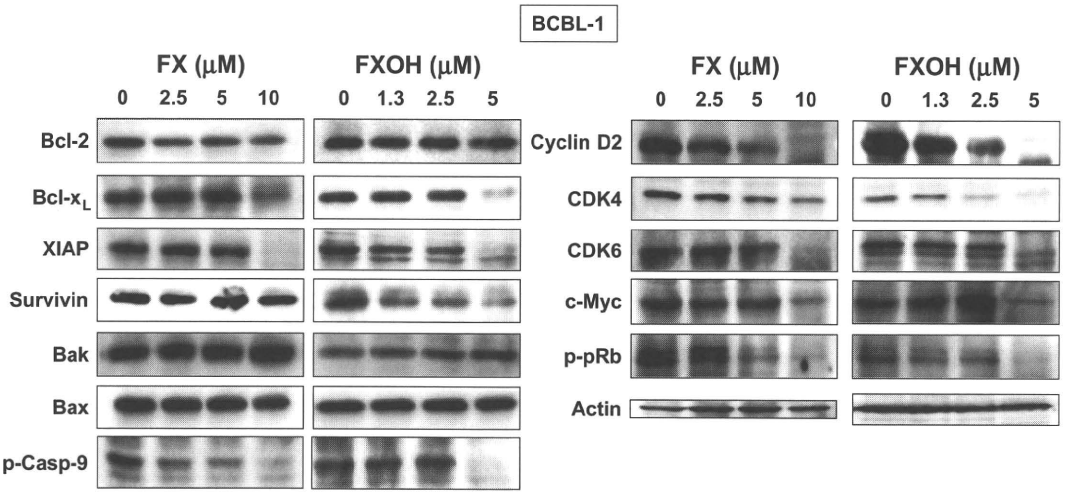

Fig. 4. Effects of FX and FXOH on the expression of apoptosis and cell cycle regulatory proteins. BCBL-1 cells were treated with the indicated concentrations of FX or FXOH for 24 h. Protein levels were detected by Western blotting with antibodies directed against each protein.

in BCBL-1 and TY-1 cells (Fig. 2C). These results demonstrate that caspase activation plays a role in the FX- and FXOH-induced apoptosis observed in the PEL cell lines.

3.4. FX and FXOH cause G_1 cell cycle arrest

We next examined the effects of FX and FXOH on the cell cycle regulating mechanisms using BCBL-1 and TY-1 cells. Cultivation with FX and FXOH for 24 h increased the population of cells in the G_1 phase, with marked reduction of cells in the S phase, relative to untreated cells (Fig. 3). Thus, FX and FXOH suppressed the proliferation of PEL cell lines by arresting the cells in the G_1 phase of the cell cycle.

3.5. Effects of FX and FXOH on the expression of apoptosis and cell cycle regulatory proteins

To clarify the molecular mechanisms of FX- and FXOH-induced inhibition of growth and apoptosis of PEL cells, we investigated the effects of FX and FXOH on the expression of several intracellular regulators of cell cycle and apoptosis by Western blot analysis. As shown in Fig. 4, FX and FXOH did not alter the expression levels of anti-apoptotic protein Bcl-2, or pro-apoptotic proteins Bak and Bax. In contrast, FX and FXOH down-regulated the expression levels of anti-apoptotic proteins Bcl-x_L and XIAP in a dose-dependent manner. FXOH also inhibited the expression of survivin, an anti-apoptotic protein. Since pRb functions as a regulator of cell cycle progression in the late G_1 phase [36], we studied the effect of FX and FXOH on pRb phosphorylation. As shown in Fig. 4, FX and FXOH reduced the phosphorylation of pRb protein after treatment. Furthermore, FX and FXOH down-regulated the expression levels of cell cycle regulatory proteins cyclin D2, CDK4, CDK6 and c-Myc in a dose-dependent manner. Our results demonstrated that FX- and FXOH-mediated growth inhibition and apoptosis was associated with reduced expression of Bcl-x_L, XIAP, survivin, cyclin D2, CDK4, CDK6 and c-Myc in PEL cell lines.

3.6. Inhibitory effects of FX and FXOH on NF-κB, AP-1 and PI3K/Akt pathways

Because Bcl-x_L, XIAP, survivin, cyclin D2, CDK4, CDK6 and c-Myc are NF-κB target genes [37–41], we examined whether FXOH inhibits the NF-κB pathway. To study the DNA-binding activity of NF-κB, we performed EMSA with radiolabeled double-stranded NF-κB oligonucleotides and nuclear extracts from BCBL-1 cells. The results confirmed the constitutive activation of NF-κB. Supershift analysis showed that NF-κB bands were composed of p50 and p65 (Fig. 5A, left panel). We next examined the effects of FXOH on BCBL-1 cells. Results of EMSA demonstrated loss of DNA binding of NF-κB in a time-dependent manner (Fig. 5B), suggesting that FXOH could inhibit the DNA-binding activity of NF-κB. Immunoblotting showed that in the absence of FX or FXOH, the levels of phosphorylated IKKβ and IκBα steadily increased in BCBL-1 cells. FX and FXOH reduced the phosphorylation of IKKβ and IκBα in a dose-dependent manner. Interestingly, FX and FXOH reduced the levels of IKKα, IKKβ and IKKγ (Fig. 5C, left panels).

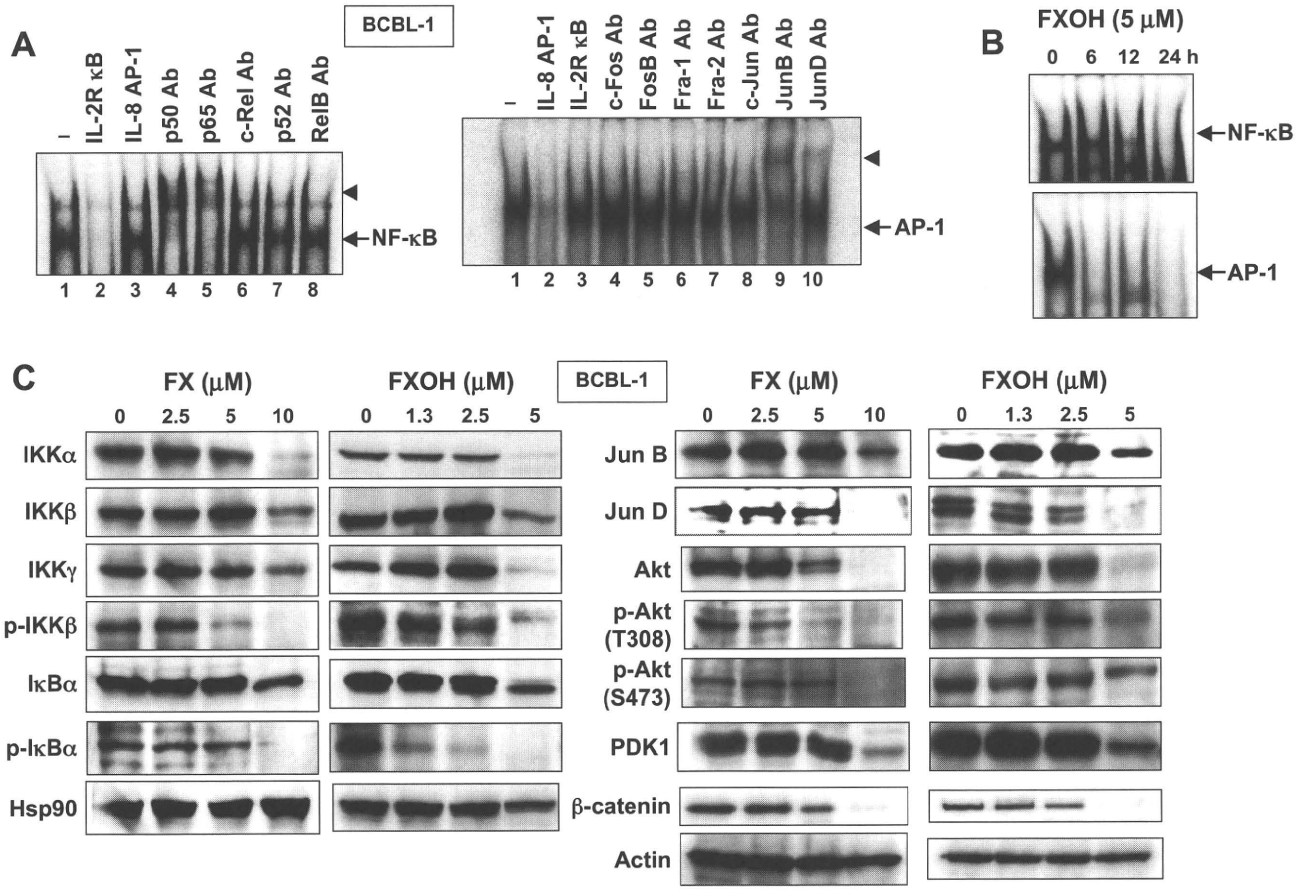

Fig. 5. FX and FXOH inhibit NF-κB, AP-1 and PI3K/Akt pathways. (A) Abundant constitutive NF-κB and AP-1 DNA-binding activity in BCBL-1 cells. EMSA using untreated BCBL-1 nuclear extracts and radiolabeled NF-κB and AP-1 probes generated DNA–protein complexes, which were eliminated by 100-fold molar excess of self-competitors but not by the same molar excess of the irrespective oligonucleotides. Supershift assays using the radiolabeled NF-κB and AP-1 probes, untreated nuclear extracts, and the indicated antibodies (Ab) to NF-κB and AP-1 components showed that the NF-κB and AP-1 bands consisted of p50 and p65 subunits, and JunB and JunD subunits, respectively. Arrows: the specific complexes, arrowheads: the DNA binding complexes supershifted by antibodies. (B) Effect of FXOH on NF-κB and AP-1 binding activities. BCBL-1 cells were treated with FXOH (5 μM) for indicated time intervals. Nuclear extracts were examined for NF-κB and AP-1 binding activities by EMSA. (C) FX and FXOH inhibit the phosphorylation of IKKβ and IκBα, and reduce the amounts of IKKα, IKKβ, IKKγ, Akt and PDK1 proteins. BCBL-1 cells were treated with the indicated concentrations of FX or FXOH for 24 h, followed by protein extraction. Whole cell extracts of treated cells were immunoblotted with specific antibodies against each protein.

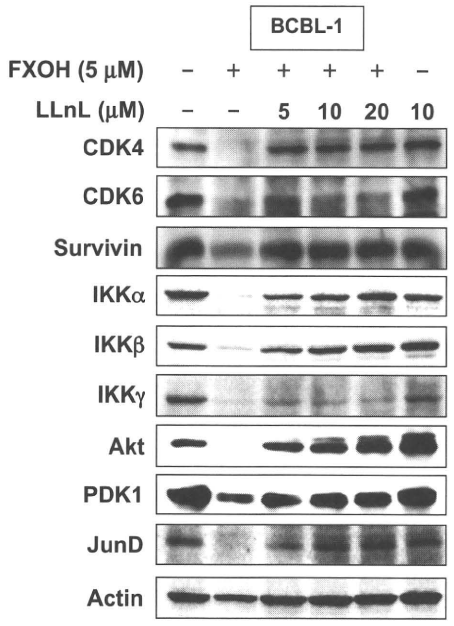

Fig. 6. Proteasome degradation is responsible for decreased levels of proteins after FXOH treatment. BCBL-1 cells were treated either with various concentrations of proteasomal inhibitor LLnL in the presence or absence of FXOH (5 μM) for 24 h, or left untreated, as indicated. Samples were analyzed for each protein by Western blotting.

In the next step, EMSA was used to determine whether FXOH modifies the activity of AP-1, a transcription factor associated with PEL cell growth [16]. BCBL-1 cells showed high levels of AP-1 constitutive activity. Supershift analysis with antibodies indicated that the AP-1 complex contained JunB and JunD (Fig. 5A, right panel). FXOH reduced the constitutive AP-1 binding in BCBL-1 cells within 6 h of treatment; and this effect was maintained for 24 h (Fig. 5B). FX and FXOH decreased JunB and JunD levels (Fig. 5C, right panels), suggesting that their inhibitory effects on AP-1 activation are at least in part mediated at the level of JunB and JunD.

The PI3K/Akt signaling pathway contributes to PEL cell survival [21,22]. The involvement of PI3K as an essential component of the survivin expression has been reported [40]. FX and FXOH reduced the amounts of PDK1 and Akt proteins, downstream kinases of PI3K in BCBL-1 cells (Fig. 5C, right panels). Akt was found to phosphorylate caspase-9 and thereby diminishes its activity [42]. A downstream target of Akt is also the proto-oncogene β-catenin [42]. FX and FXOH reduced the phosphorylation of caspase-9 (Fig. 4, left panels) and the level of β-catenin (Fig. 5C, right panels) in a dose-dependent manner.

3.7. FXOH-induced reduction of protein levels is due to proteasome degradation

To examine whether proteasomal degradation was responsible for decreased levels of proteins after FXOH treatment, BCBL-1 cells were cultured in a medium containing FXOH and proteasome inhibitor LLnL. FXOH-mediated reduction of proteins (CDK4, CDK6, survivin, IKKα, IKKβ, IKKγ, Akt, PDK1 and JunD) was blocked by LLnL (Fig. 6). This effect of LLnL suggests that these proteins are subject to ubiquitin-dependent turnover.

3.8. FX and FXOH do not induce HHV-8 reactivation

It was reported previously that NF-κB inhibition leads to replication of HHV-8 [43]. Therefore, RT-PCR was performed to examine the effects of FX and FXOH on viral gene expression. Two viral genes known as lytic genes were selected in this analysis. There was no significant increase in the expression levels of lytic genes after treatment (Fig. 7A), suggesting that FX and FXOH do not induce viral production within BCBL-1 cells.

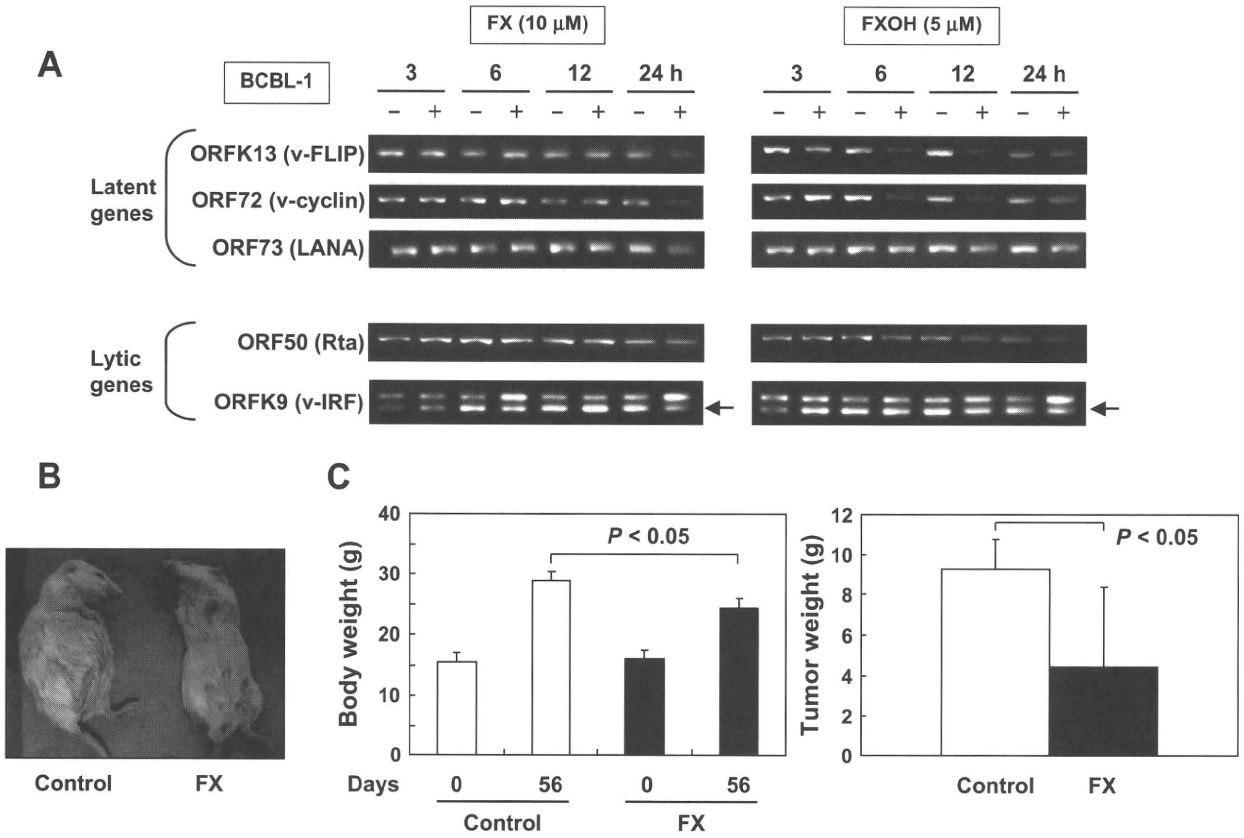

Fig. 7. (A) Viral gene expression after treatment with FX and FXOH. Viral gene expression after treatment with FX (10 μM) and FXOH (5 μM) at different time points was examined by RT-PCR. (B) A photograph of FX-treated and untreated SCID mice on day 56 after cell inoculation. (C) Inhibition of growth of BCBL-1 cells in SCID mice. Body weight of mice ($n = 3$, left panel) and weight of tumors (right panel) removed from FX-treated ($n = 3$) and untreated mice ($n = 3$) on day 56 after cell inoculation. Data are mean ± SD.

Importantly, the expression levels of v-FLIP and v-cyclin, but not LANA, were down-regulated after 24 h of FX treatment and after 6–12 h of FXOH treatment (Fig. 7A). Taken together, FX and FXOH do not seem to induce virus proliferation, although they down-regulated the expression of latent genes such as v-FLIP and v-cyclin.

3.9. In vivo effects of FX in SCID mice inoculated with PEL cells

Finally, we examined whether FX treatment is effective against xenografted tumors in a SCID mice model. The gross appearance of mice treated with or without FX was different; abdominal distention was less in FX-treated mice than in untreated mice (Fig. 7B). After 56-day treatment, the mean body weight of mice and weight of tumor were significantly lower than those of untreated mice (Fig. 7C). Body weight was higher in the control group, which was explained by the fact that untreated mice developed tumors and effusion in body cavities. These results demonstrated that FX is effective against PEL cells in mice, suggesting that it could be potentially suitable therapeutically in patients with PEL.

4. Discussion

PEL is recognized as a unique clinical entity comprising of HHV-8-infected transformed post-germinal center B cells. PEL is a very aggressive type of lymphoma and is unusual in that the majority of cases arise in body cavities, such as the pleural space and the pericardium. It is generally resistant to chemotherapy that is otherwise potent against other lymphomas [5]. Therefore, there is a need for the development of new therapies. In this study, we demonstrated that FX and FXOH inhibit the growth of PEL cells both *in vitro* and *in vivo*. It was noteworthy that FX and FXOH had few adverse effects on normal cells both *in vitro* and *in vivo*. Our results showed that both FX and FXOH had potent pro-apoptotic effects on PEL cells and suggest that these agents may prove to be of value in the treatment of PEL.

Previous studies showed that individual HHV-8 viral proteins activate the NF-κB, AP-1 and PI3K/Akt pathways [7–12,16–20,23–25]. HHV-8-infected PEL also displays constitutively high activity of the NF-κB, AP-1 and PI3K/Akt pathways [14,17,18,21,22]. FX can also induce growth inhibition and apoptosis in several cell lines [26,29]; however, the effective concentration differ among the cell lines, and the precise mechanism of its action remains uncertain. Our results of Western blot analysis confirmed down-regulation of many target genes in these pathways such as anti-apoptotic and cell cycle progression genes in PEL cells. Furthermore, the present work demonstrated that FX and FXOH inhibited the activation of these three pathways in PEL cell lines. Apoptotic cell death was observed without reactivation of HHV-8. Thus, FX and FXOH seem to be promising candidates for molecular targeting therapy of PEL.

In the present study, we explored the potential mechanisms of the inhibitory effects of FX and FXOH on the activation of NF-κB, AP-1 and PI3K/Akt pathways in PEL cells. Hsp90 displays a chaperoning function for unstable signal transducers to keep them poised for activation, although it is not required for their maturation or maintenance [44,45]. Hsp90 directly interacts with client proteins and forms a mature complex that catalyzes the conformational maturation of Hsp90 client proteins [46,47]. Several Hsp90 client proteins are degraded by the proteasome following Hsp90 inhibitors, and CDK4, CDK6, survivin, IKKα, IKKβ, IKKγ, Akt and PDK1 are client proteins [46–48]. Although FX and FXOH did not affect the level of Hsp90 (Fig. 5C), FXOH depletes Hsp90 client proteins in PEL cells. Furthermore, FXOH-mediated reduction of proteins was blocked by LLnL (Fig. 6). These results suggest that FX and FXOH inhibit the function of Hsp90 chaperone in PEL cells.

FX and FXOH did not induce transition from the latent to lytic phase of HHV-8. In contrast, the latent viral genes such as v-FLIP and v-cyclin, showed reduced gene expression levels. v-FLIP is essential for the survival of PEL cells while v-cyclin interferes with cell cycle deregulation [11,12,49]. Inhibition of expression of v-FLIP and v-cyclin may lead to the induction of growth inhibition and apoptosis by FX and FXOH.

Orally administered FX is a safe compound in terms of mutagenicity [29]. Dietary FX is considered to be hydrolyzed to FXOH in the gastrointestinal tract by digestive enzymes such as lipase and cholesterol esterase and then absorbed into intestinal cells [50]. It was also reported that FXOH is a major metabolite of dietary FX in humans, indicating that the major compound in the circulations after FX intake is FXOH [51]. The present study using PEL cell lines showed that the apoptosis-inducing activity of FXOH was more potent than that of FX. Taken together, most dietary FX may be converted to FXOH, and FXOH may exert a suppressive effect on PEL cells at concentrations lower than the effective concentrations of FX used in the present study.

In conclusion, FX and FXOH effectively induced cell growth arrest and apoptotic cell death in PEL cells at a concentration nontoxic to normal cells and these effects may be associated with functional inhibition of Hsp90 chaperon. In addition, FX demonstrated anti-PEL effect in the xenografted mice. These results suggest that dietary FX or FXOH could be potentially helpful for the treatment of PEL.

Conflict of interest

None declared.

Acknowledgment

We acknowledge Dr. Shigeki Sawada for the expert technical assistance.

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