Pathology (October 2020) **52(6)**, pp. 676–685

ANATOMICAL PATHOLOGY

Epstein–Barr virus is a promoter of lymphoma cell metastasis

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Summary

It is well-known that Epstein–Barr virus (EBV) is the promoter of cell tumourigenesis. We found that EBV is also a promoter of lymphoma cell dissemination, because we found the typical morphopathological phenomenon of cell adhesion, which confirmed that the adhesion of tumour cells was higher than that of normal cells. We also observed that tumour cells disrupted the dynamic pathological changes of vascular endothelial cells, and this made it clear that the rate of tumour cell metastasis was directly proportional to the degree of EBV infection. Furthermore, when we discovered exosomes, it was considered that this was associated with cancer stem cells, suggesting the formation of a microenvironment before tumour cell metastasis.

In addition, competitive inhibition was found in cell adhesion, indicating the breakthrough point of preventing tumour cell metastasis, which has clinical reference value for tumour immunotherapy.

Key words: Cell metastasis; Epstein–Barr virus; lymphoma; adhesion molecule; competitive inhibition.

Received 22 January, revised 3 May, accepted 13 May 2020
Available online 4 August 2020

INTRODUCTION

Epstein–Barr virus (EBV) was discovered in 1964 by Epstein, Achong and Barr in the cells of patients with Burkitt's lymphoma. It is known that EBV belongs to the γ subfamily of human *Herpesviridae*, and is a double-stranded filamentous DNA virus covered with glycolipoproteins.[1] Furthermore, it is spherical, with a diameter of 180–200 nm. In addition, its nucleic acids manifest as linear double strands of DNA, and its length varies with the different strains of the virus, with an average length of 175 kb. This virus is encapsulated by capsid protein. EBV infects human cells through the glycoprotein gp350/220 on its envelope that binds with lymphocyte CR2.[2]

EBV is mainly transmitted through saliva or blood transfusion.[3] It first targets B cell receptors via the cell membrane glycoprotein, and infects B lymphocytes.[4] The exosome secreted by B lymphocytes has an antigen presenting ability.[5] It presents tumour antigens to cytotoxic T lymphocyte (CTL), activates T lymphocytes, and subsequently induces a series of pathological changes, such as cell gene mutation and cytokine response.

When these cells become cancerous, these continue to deteriorate, and finally, cell metastasis occurs. Statistics have shown that 90% of tumour patients die of tumour metastasis.[6] Therefore, there is an urgent need to analyse the principle of tumour cell metastasis.

There are many different interpretations regarding the cause of tumour cell metastasis. First, some scholars proposed a simple theory of 'anatomy and mechanics', then some experts proposed a fusion theory.[7] Subsequently, according to the hypothesis of the relationship between the 'seed' and 'soil' of tumour metastasis, the formation mechanism of the microenvironment before tumour metastasis was proposed.[8] In recent years, this theory has further progressed into the tumour stem cell theory,[9] which explains the genetic heterogeneity of tumour metastasis, the resistance to nest loss and apoptosis, tumour dormancy and other phenomena.[10] However, we speculated that cell metastasis is closely correlated to cell adhesion molecules. Therefore, we used non-Hodgkin's lymphoma cells obtained from 14 patients, and human tonsil vascular endothelial cells (TEVC) and skin vascular endothelial cells, in order to carry out a cell adhesion test.

We attempted to test and confirm the following points through experiments: Is EBV correlated to tumour metastasis? Does the degree of EBV infection affect the rate of tumour cell metastasis? What about the static and dynamic manifestations of cell adhesion? What role does adhesion molecule play in cell metastasis? Do the exosomes secreted by tumour cells affect cell metastasis? What is the impact of latent membrane protein 1 (LMP-1) on cell metastasis? The aim of discussing all these questions was to understand the principle of tumour cell metastasis, and find a way to stop tumour cell metastasis.

MATERIALS AND METHODS

Lymphoma cells

Non-Hodgkin lymphoma B cells obtained from 12 patients were selected and prepared in a tumour-loaded severe combined immunodeficiency (SCID) mouse model. These were divided into two groups and underwent subculture: EBV-positive group (SMS-6, TMS-7, TMS-10, AMS-12, NMS-15 and SMS-16) and EBV-negative group (SMS-1, YMS-2, AMS-3, HMS-11, KMS-14 and HMS-24). Then, the tumour tissues that subcutaneously grew in mice were prepared into frozen sections. In addition, two cases of lymphoma tissue were selected to establish two B lymphoma cell lines, which were code-

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2020.05.005

named as TC-1 (EBV+) and SD-1 (EBV−), and subcultured. Then, the long-term adhesion between these tumour cells and vascular endothelial cells was observed. We used SCID mice of one month old. This animal model was a tumour bearing (lymphoma) mouse which was passaged monthly, thereby it had also become a living lymphoma cell line. When the subcutaneously transplanted tumour grew to 0.5 cm in diameter, this was taken out for use. The source of the normal human lymphocytes was the Department of Pathology, Graduate School of Medicine, The University of Tokyo.

Vascular endothelial cells

We used cryopreserved sections of normal human tonsil vascular endothelial cells (TVEC) and the primarily cultured cell line of normal human dermal microvessel vascular endothelial cells (MVECs, which passed the IRB review of Tokyo University). The cell culture media included RPMI 1640 medium (GIBCO-BRL, USA), 15% (V/V) fetal calf serum (FCS), 1 mL of 50 μg/mL gentamicin, 50 μg/mL of platelet-derived endothelial cell growth factor (PD-ECGF), 10 ng/mL of endothelial growth factor (EGF; Cosmo Bio, Japan) and 50 μg/mL of heparin. The medium was added with 100 U/L of penicillin and 100 mg/L of streptomycin, and cultured in an incubator with 5% CO_2 at 37°C.

Establishment of primary culture cell line

First, sections of the tumour tissue were placed in a small bottle, rinsed three times with Hank's balanced salt solution, and repeatedly cut with ophthalmic scissors into small pieces with a size of approximately 1 mm.[3] Trypsin (Nichirei Corporation, Japan), with an amount of 30–50 times more than the total amount of tissue pieces, was added. Then the mixture was placed into a triangular flask, digested in a temperature box at 37°C, shaken once every 20 min, and centrifuged at a low speed (1000 rpm) for 5 min. Next, the supernatant containing a single cell suspension was obtained and placed in the cell culture medium (Sigma-Aldrich, USA), allowing the cells to survive and reproduce. Finally, the EBV-positive TC-1 and EBV-negative SD-1 tumour cell lines were established.

Immunohistochemical staining

On the frozen sections of lymphoma tissues, and the sections fixed with formalin and embedded with paraffin, α-LMP-1 antibody (CS1-4; Dako, Japan), α-lymphocyte function-associated antigen 1 (α-LFA-1) antibody (CD11a; Nichirei Corporation) and αICAM-1 antibody (CD54; Dako) were used as the primary antibodies for treatment. Biotinylated anti-mouse immunoglobulin (Dako) was used as the secondary antibody. Strip avidin conjugated with LAB-peroxidase (Dako) was used for the third reaction. An AEC kit (Nichirei Corporation) was used for colouration. Some sections were stained with fluorescence, dropped with the fluorescein-labelled second antibody matched with the primary antibody species (goat anti-mouse FITC-IgG; 1:100; Merck & Co, USA), placed in the dark and incubated at 37°C for 30 min. Finally, the specimens were sealed with a water-soluble mediator (Biomeda Corporation, USA) and observed under a light microscope (Leitz DM RXE; Leica Microsystems, Germany).

In situ hybridisation

The frozen sections were fixed with paraformaldehyde, treated with 0.2 N HCl, and treated with 10% streptomycin protease (Sigma-Aldrich). These were subsequently incubated with buffer for 2 h, and treated with 0.25% acetic anhydride (Sigma-Aldrich). The anti-digoxin antibody labelled with alkaline phosphatase was used for the colouration, and nitro blue tetrazelium/5-bromo-4-chloro-3-indenyl-phosphate (NBT/BCIP; Promega, USA) was used as the colour source. In addition, 0.1% diethyl-pyrocarbonate (DEPC; Nippon Gene Co, Japan) was used for treatment. The EBV-encoded small RNA-1 (EBER-1) probe was synthesised using a DNA synthesis device (Cyclone; Sigma-Aldrich, USA). The device consisted of 30 bases (5′-AGA CAC CGT CCT CAC CCG GGA CTT GTA-3′). The probe was used at a concentration of 10–100 μg/mL. The EBER-1 probe was labelled according to digoxin 3′ end labelling method. The expression of the EBER-1 gene in the nucleus of tumour cells was detected.

Cell adhesion test

1. At T = 0 minute, 0.1 mL of lymphoma cell suspension (about 100 cells) was added to the frozen TVEC section, and the adhesion of tumour cells was observed at T = 30, 60, 90 and 120 min under an optical microscope.
2. At T = 0 minute, 0.1 mL of suspension of lymphoma cells was added to the culture medium of MVEC, and the adhesion of tumour cells was observed at T = 1, 2, 4, 6, 12, 24, 48 and 72 min under an optical microscope.
3. 0.1 mL of suspension of TC-1 and SD-1 tumour cells were added to TVEC and MVEC, respectively, in order to observe the adhesion profile of each type of cell, respectively.
4. The intercellular adhesion molecule 1 (ICAM-1) antibody was diluted 100 times and incubated with TVEC at warm temperature for 1 h. Then the samples were rinsed, and the tumour cells were added to TVEC, in order to observe the adhesion of these cells to TVEC.
5. Normal human lymphocytes were added to TVEC as the control group.
6. The SPSS statistics method (SPSS; IBM, USA) was used to analyse the binding of EBV-positive and EBV-negative cells to vascular endothelial cells, and the different binding rates of EBV-infected lymphoma cells were detected.

Electron microscopy

We utilised the transmission electron microscope at the Institute of Urban Environment of the Chinese Academy of Sciences (Xiamen) to establish the cell hybridisation-ultrastructure detection method, and observe the pathological changes of the ultrastructure of these cells.

Specific operation steps were as follows. First, the lymphoid tissue on the glass piece was rehydrated with a diminishing concentration gradient of ethanol, fixed with 1% osmium tetroxide, rinsed and dehydrated with an increasing concentration gradient of ethanol, and embedded with Spurr resin. Then, the mass was sliced into sections on the Leica UC7 ultramicrotome after dewaxing by heating, and stained with uranyl acetate and lead citrate. Finally, the sections were observed under a H-7650 transmission electron microscope (Hitachi, Japan).

We also used the S-4800/FE-electron microscope (Hitachi) at the Department of Pathology, Hamamatsu Medical University, Japan, to observe the cells. After coating with nano spraying solution, the samples were diluted using a rotary coating machine at an accelerating voltage of 1.0–5.0 kV (the vacuum level of the observation chamber was approximately 10^{-3} to 10^{-6} Pa), and the detector at the lower position was used to perform the secondary electron scanning.[11]

RESULTS

Expression of adhesion molecule, LMP-1 and emergence of exosomes

In the study of tumour cell metastasis, considering that the adhesion between tumour cells and cells must be mediated by adhesion molecules, we first adopted non-Hodgkin's lymphoma cells, and these cells were subcutaneously cultured in SCID mice. After 1 month, the tumour tissue was taken out and made into frozen sections for immunohistochemical staining. Results are as follows. In six specimens of EBV-positive tumour tissues (Fig. 1A; SMS-6, TMS-7, TMS-10, AMS-12, NMS-15 and SMS-16), five specimens (83%) exhibited positive LFA-1 expression, and a large amount of LFA-1 existed in the germinal centre of lymph nodes (Fig. 1B). ICAM-1 was positive in six specimens (100%), and a large amount of ICAM-1-positive cells were observed in the dark zone of lymph nodes (Fig. 1C). LMP-1 was positive in five specimens (83%), and in these bud-forming specimens (Fig. 1D), one specimen was negative for LMP-1 (Table 1). In the other six specimens of EBV-negative tumour tissues (SMS-1, YMS-2, AMS-3, HMS-11, KMS-14 and HMS-24) five specimens were negative for LFA-1, one specimen was weakly positive for LFA-1, two specimens were positive for ICAM-1 (33%), and six specimens were negative for LMP-1 (Table 1).

LFA-1 (Fig. 2A), ICAM-1 and LMP-1 were positive in the TC-1 tumour cell line (Table 1). In the SD-1 tumour cell line, LFA-1 (Fig. 2B) was negative, ICAM-1 was weakly positive, and LMP-1 was negative (Table 1).

Exosomes were found in cells by transmission electron microscopy (Fig. 2C), suggesting the possibility of the

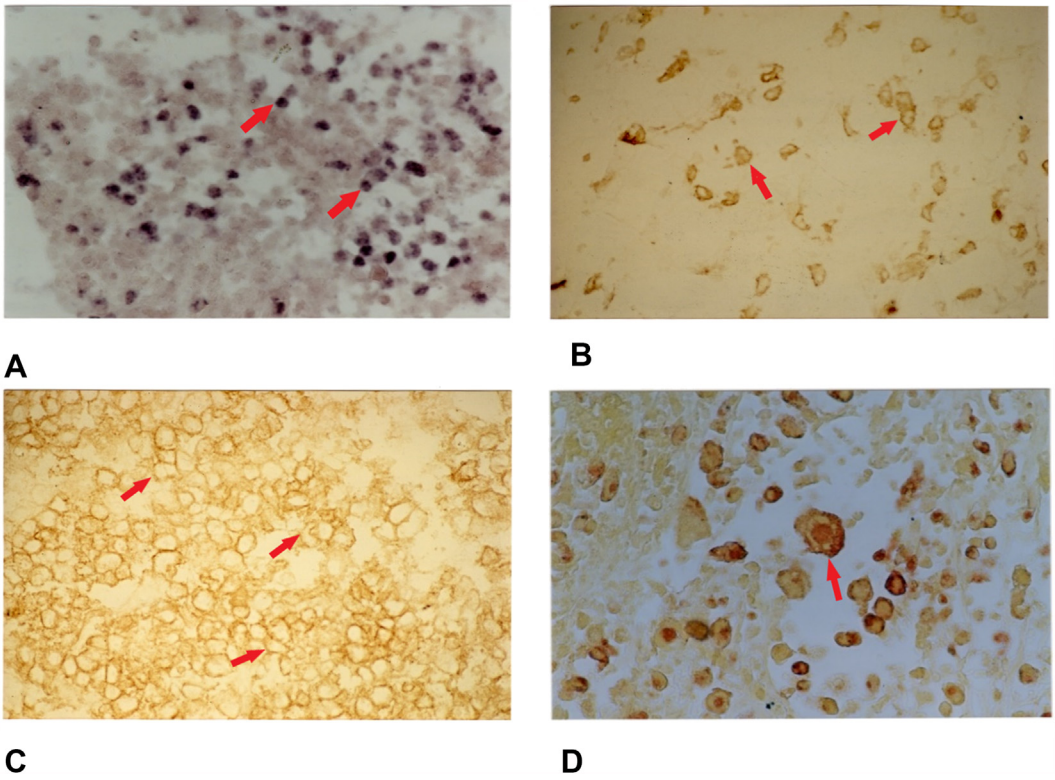

Fig. 1 (A) In the specimens obtained from patients with malignant lymphoma, the expression of EBER-1 was found. The red arrow points to the black nucleus, indicating the presence of EBV particles in tumour cells. (B) LFA-1 positivity was confirmed in the germinal centre area of lymph nodes of EBV(+) lymphoma tissue. (Tumour cells indicated by red arrows.) (C) ICAM-1 positivity was confirmed in the dark zone of lymph nodes in EBV(+) lymphoma tissue. (Tumour cells indicated by red arrows.) (D) After the *in situ* hybridisation of cells, it was revealed that EBER-1 was expressed in the nucleus. LMP-1 was positive in the immunohistochemical staining. The red arrow indicates that the cell has undergone a nucleus enlargement. These results indicate that when LMP-1 appears in EBV-infected cells these cells sporulate.

formation of the microenvironment before tumour cell metastasis.

High adhesion of tumour cells with vascular endothelial cells

In the examination of EBV-infected lymphoma tissues by scanning electron microscope, it was found that there was tumour cell adhesion in the microvasculatures (Fig. 3A,B). In order to further verify the accuracy of these pathological changes, we used frozen human TVEC to contact the cells of the SCID mouse lymphoma cell line. After 30 min, the tumour cells began to adhere to vascular endothelial cells. Then, after 60 min, the adhesion phenomenon reached its peak (Fig. 3C). Typical morphological and pathological changes of tumour cell metastasis were discovered for the first time. The adhesion rate of EBV-positive cells was 50% ($p<0.01$, Fig. 3E). In the control group, no adhesion of EBV-negative lymphoma cells was observed (Fig. 3D).

Among the six EBV-positive tumour cell lines (SMS-6, TMS-7, TMS-10, AMS-12, NMS-15 and SMS-16), all of these (100%, 6/6) adhered to the vascular endothelial cells. Among the six EBV-negative tumour cell lines (SMS-1, YMS-2, AMS-3, HMS-11, KMS-14 and HMS-24), merely one of these (17%, 1/6) significantly adhered with vascular endothelial cells (Table 2), but no adhesion phenomenon was found in the other five cell lines.

In order to observe the process of tumour cell metastasis for a long period of time, we used cultured MVEC from human dermal tissues to contact the cultured TC-1 lymphoma cell line. After one hour, TC-1 tumour cells began to adhere to MVECs, the tumour cells invaded into the cell membrane of MVECs after 48 hours (Fig. 3F), and the MVECs were destroyed after 72 hours (Fig. 4A). The highest adhesion rate was 75% ($p<0.01$, Fig. 4B). Subsequently, these tumour cells continuously increased, and the MVECs were constantly destroyed and reduced until these disappeared (Fig. 4C–E), while the adhesion ability of the SD-1 cell line was poor.

In addition, no normal human lymphocytes adhered with the vascular endothelial cells (Table 2).

Briefly, the adhesion abilities of the cultured tumour cell lines were stronger than those of tumour cells obtained from SCID mice, and the TC-1 cell line had the highest adhesion ability.

Vascular endothelial cells reject the adhesion of tumour cells

According to the principle of antigen-antibody reaction, TVECs were treated with the ICAM-1 antibody. Then, these were added with lymphoma cells, and the receptor-ligand reaction between tumour cells and vascular endothelial cells was observed. After one hour, no adhesion between these two was found (Fig. 4F). This shows that there were new changes in the cell membrane; that is, when the antigen-antibody binding occurred on the cell membrane, the vascular endothelial cells were in a saturated state and the activity of the receptors was lost. In refractory stage, receptor-ligand reactions did not occur. This is the phenomenon of the competitive inhibition of cells.

Table 1 Expression of LFA-1, ICAM-1 and LMP-1 in the different specimens

Specimen	Case no.	LFA-1	ICAM-1	LMP-1
SCID mouse EBV(+) tumour cell (frozen section)	SMS-6	+	+	+
	TMS-7	−	+	−
	TMS-10	+	+	+
	AMS-12	+	+	+
	NMS-15	+	+	+
	SMS-16	+	+	+
SCID mouse EBV(−) tumour cell (frozen section)	SMS-1	−	+	−
	YMS-2	±	+	−
	AMS-3	−	−	−
	HMS-11	−	−	−
	KMS-14	−	−	−
	HMS-24	−	−	−
B lymphoma cell line	TC-1 EBV(+)	+	+	+
	SD-1 EBV(−)	−	±	−

Non-Hodgkin lymphoma B cells obtained from 14 patients were selected.
−, negative; ±, weak positive <10%; +, positive >10%; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function related antigen-1; LMP-1, latent membrane protein-1.

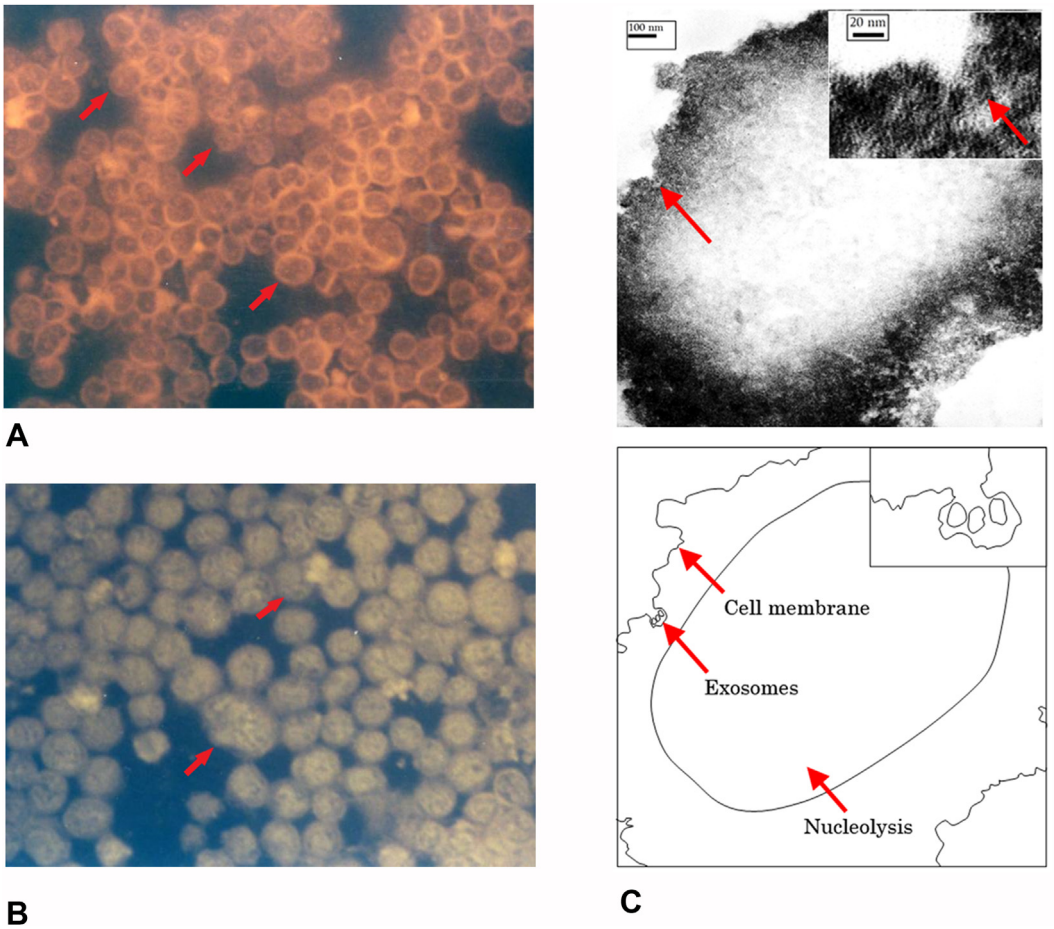

Fig. 2 (A) Expression of LFA-1 is shown on the membrane of EBV(+) and LMP-1(+) TC-1 lymphoma cell line. The red arrow indicates cultured lymphoma cells. LFA-1 expression was present in the field of fluorescence immunostaining, and it was considered to have a significant correlation with EBV infection. (B) Expression of LFA-1 was absent on the membrane of the EBV(−) and LMP-1(−) SD-1 lymphoma cell line. However, there was a positive reaction of LFA-1 in the cytoplasm (red arrow). (C) Exosomes were observed in the cell membrane depression of EBV(+) lymphoma cells (red arrow in the upper right corner points to three exosome vesicles with obvious follicular membrane). In addition, cell nucleus fragmentation was observed, the nucleolus was not found, and the nuclear membrane was indistinct (sections were embedded with paraffin, sliced and treated with electron staining) (×50,000, TEM).

DISCUSSION

We consider that in order to explain the metastasis of tumour cells, the adhesion between tumour cells and normal cells should be first investigated, and the adhesion rate should be understood, because the higher the adhesion rate is, the greater the possibility of metastasis becomes. Therefore, we began to detect adhesion molecules LFA-1 and ICAM-1[12,13] in the tumour cells of malignant lymphoma cell lines of SCID

mice[14] and their adhesion rate. The result revealed that in static pathology, the tumour cells adhered to TVECs, while in cell dynamics, these tumour cells not only adhered to MVECs, but also destroyed the MVECs. This suggests that tumour cells can be transferred to other tissues through the vascular wall.

In addition, the result revealed that the adhesion rate of EBV-positive tumour cells (75%) was higher than that of EBV-negative tumour cells (5%). Accordingly, these results confirm that EBV is a promoter of tumour cell metastasis.

At present, most scholars have discussed the metastasis of solid tumour cells, and the result of the present study revealed that there was also dissemination in lymphoma cells, especially for EBV-positive cells.

EBV is the activating factor of adhesion molecules

LFA-1 and ICAM-1 are the most representative adhesion molecules in the study of lymphoma cell dissemination. In particular, LFA-1 and ICAM-1 are strongly expressed after EBV infection. LFA-1 belongs to the integrin family, which is distributed on the surface of T and B lymphocytes, monocytes, macrophages and neutrophils. ICAM-1 is a member of the immunoglobulin superfamily, which is distributed on the surface of fibroblasts, endothelial cells and activated lymphocytes. LFA-1 and ICAM-1 can provide synergistic stimulation signals to promote lymphocyte activation, proliferation and differentiation.[15]

Lymphocytes at rest can express a certain level of LFA-1 and ICAM-1. NK cells and some CTL cell lines express higher levels of LFA-1/ICAM-1, but they do not agglutinate. When patients are infected with EBV, lymphocytes are activated, and adhesion molecules may increase the affinity between LFA-1 and ICAM-1 by changing configuration, which accordingly improves the adhesion ability of cells, finally resulting in cell metastasis. This suggests that EBV is the activating factor of adhesion molecules.

LFA-1 and ICAM-1 exhibited high adhesion to lymphoma cells. In particular, the positive rate of LFA-1 was 83% in EBV-positive cells (Table 1), which has high specific adhesion to MVECs. This was due to the continuous EBV infection, which in turn, continuously reduced the immunity and promoted the production of LMP-1 on the cell membrane.[16] Under this condition, the cells underwent spheroidisation (Fig. 1D) and immortalisation.[17] Furthermore, the Bcl-2 gene and proto-oncogene expression were induced, and the expression of p53 was downregulated. Due to the direct or indirect regulation of cell genes, the signal system of host cells was affected.[18] These experimental results revealed that the inhibition of LMP-1 expression was the key to prevent tumour metastasis.

We also observed that the EBV-negative SD-1 cell line had little adhesion ability to tumour cells. This was because LFA-1 was not expressed on the tumour cell membrane of the SD-1 cell line, but existed in the cytoplasm (Fig. 2B). Therefore, this could not bind with ICAM-1. Another possible reason is that with the absence of LMP-1, the signal transduction pathway could not be activated. Therefore, there was no cell metastasis. From this, it was found that the adhesion molecules on EBV-positive tumour cells had a better binding ability than those on EBV-negative tumour cells. Indeed, the

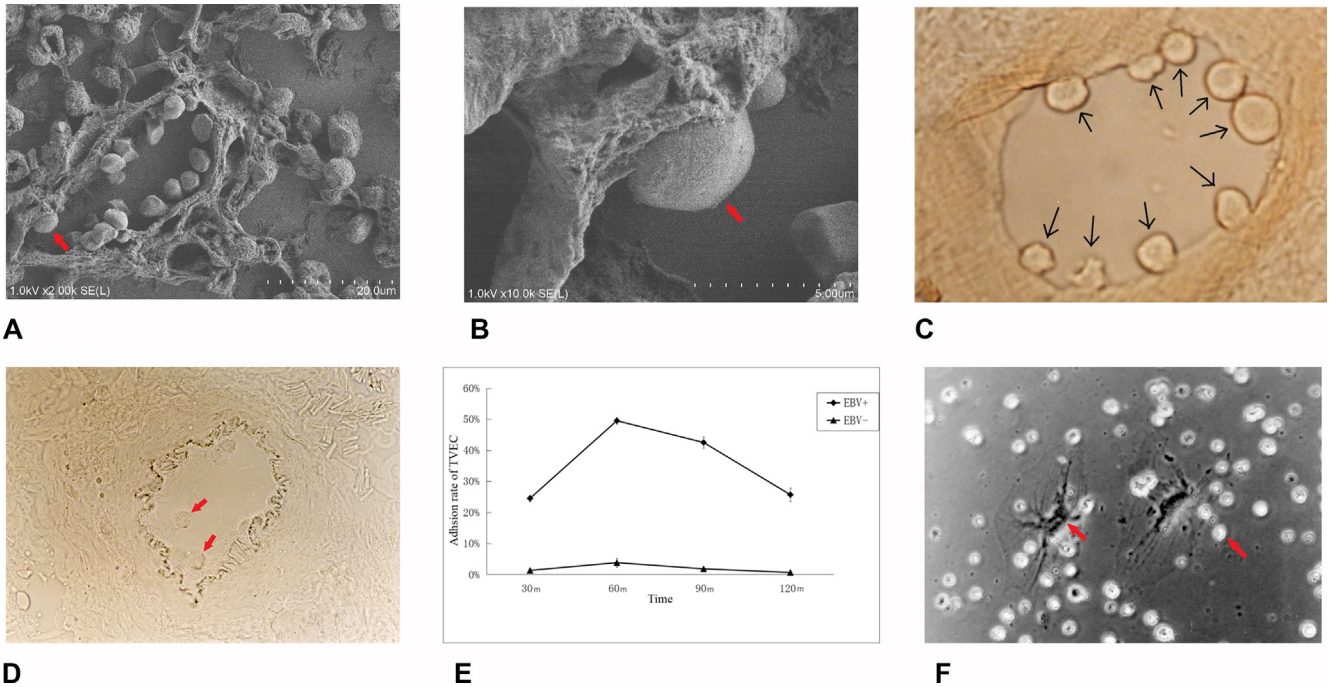

Fig. 3 (A) Lymphoma tissue was specially treated with nanofluids. Under scanning electron microscopy, tumour cells in microvessels were found to be deformed in shape and size, and some tumour cells adhered to the vessel wall (red arrow indicates tumour cell) (×2000, SEM). (B) Enlarged view of A. Red arrow indicates morphological changes in tumour cells that enter the vascular endothelium (×10,000, SEM). (C) In order to verify the accuracy of A, adhesion tests were conducted on cultured cells. After 60 min the typical adhesion state of EBV(+) lymphoma cells with tonsil vascular endothelial cells (TVECs) was observed (black arrows indicate tumour cells). (D) In the control group, no adhesion of EBV(−) lymphoma cells was observed (red arrows indicate tumour cells). (E) The cell adhesion rate was obtained by SPSS method. Calculated with 100 tumour cells, the results showed that the adhesion rate of EBV(+) lymphoma cells to TVECs was 50%, and the adhesion rate of EBV(−) lymphoma cells to TVECs was 5%. Different degrees of EBV infection led to significant differences in cell adhesion ability ($p<0.01$). (F) After 48 hours of culture of EBV(+) lymphoma cells and microvessel vascular endothelial cells (MVECs) at the same time, the adhesion of these two cells was observed. The left red arrow show the MVECs, while the right red arrow shows lymphoma cells (×200).

Table 2 Statistics of the cell adhesion between tumour cells/cell lines and TVEC/MVEC

Specimen	Case no.	EBV	TVEC adhesion	MVEC adhesion
SCID mouse tumour cell (living cell)	SMS-6	EBV(+)	++	+++
	TMS-7	EBV(+)	+	++
	TMS-10	EBV(+)	++	+++
	AMS-12	EBV(+)	++	+++
	NMS-15	EBV(+)	++	+++
	SMS-16	EBV(+)	++	+++
SCID mouse tumour cell (living cell)	SMS-1	EBV(−)	±	±
	YMS-2	EBV(−)	+	+
	AMS-3	EBV(−)	±	±
	HMS-11	EBV(−)	±	±
	KMS-14	EBV(−)	±	±
	HMS-24	EBV(−)	−	±
B cell lymphoma cell line	TC-1	EBV(+) LMP-1(+)	+++	+++
	SD-1	EBV(−) LMP-1(−)	±	±
After ICAM-1 blocking on TVEC			−	NA
Normal human lymphocyte on TVEC			−	NA

Non-Hodgkin lymphoma B cells obtained from 14 patients were selected.
−, negative; ±, positive <5%; +, positive <25%; ++, positive <50%; +++, positive <75%; ++++, positive <100%; MVEC, microvascular endothelial cell; SCID, severe combined immunodeficiency; TVEC, tonsil vascular endothelial cell.

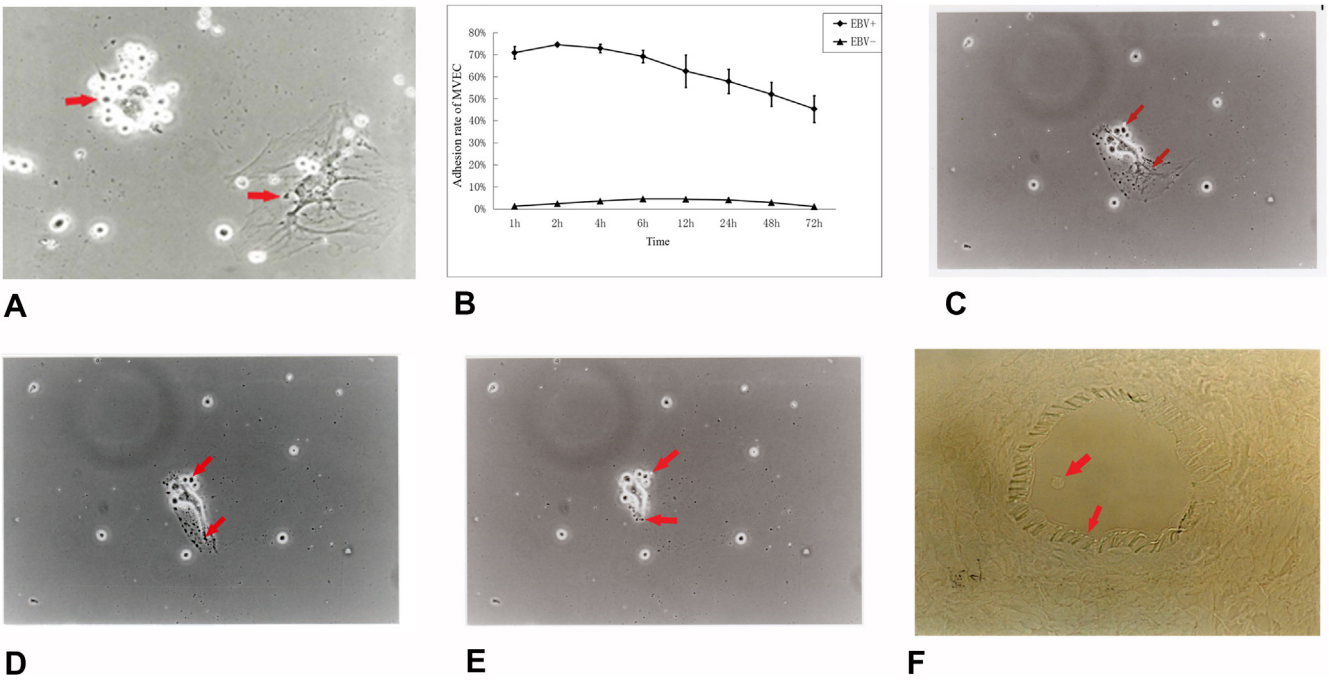

Fig. 4 (A) After 72 hours, lymphoma cells continued to adhere to and destroy microvessel vascular endothelial cells (MVECs) (the right red arrow shows MVEC, and the left red arrow shows lymphoma cells) (×200). (B) The highest adhesion rate of EBV(+) lymphoma cells to MVECs was 75%, and the highest adhesion rate of EBV(−) lymphoma cells to MVECs was 5%. The results reveal that EBV-positive cells have a strong adhesion ability ($p<0.01$). (C) The EBV(+) lymphoma cells (round floating cells) begin to adhere to MVECs (umbrella-shaped adherent cells) (×200). (D) After one minute, the same tumour cells continued to destroy the MVEC, making it gradually smaller (×200). (E) After two minutes, the MVEC almost disappeared. It can be observed in the dynamic diagram of cell adhesion that once tumour cells adhere to MVECs, these are rapidly destroyed by tumour cells (red arrow top shows tumour cells, and red arrow below shows MVEC) (×200). (F) The tonsil vascular endothelial cell (TVEC) specimen was added with the anti-ICAM-1 antibody. Then the specimen was rinsed and EBV(+) lymphoma cells were placed on the specimen. No adhesion of lymphoma cells to TVECs was found after one hour. This result reveals that this is the phenomenon of the competitive inhibition of cells (red arrow top shows tumour cells, and red arrow below shows MVECs) (×400).

adhesion of tumour cells was also prior to that of normal cells.

EBV is a promoter of tumour cell metastasis

In the present study, the whole process of adhesion of EBV-positive cells to TVECs was observed. The adhesion rate reached the maximum value after one hour. The typical adhesion state of tumour cells to vascular endothelial cells was found for the first time (Fig. 3C). The process of tumour cell migration suggests the following: (1) LFA-1 on tumour cells closely binds to ICAM-1 on vascular endothelial cells; (2) the deformation of endothelial cells and their position exchange with tumour cells; (3) tumour cells reach the extracellular matrix (ECM) through the space of the vascular endothelial cells and finally enter the extravascular area (Fig. 5). It was observed that EBV-positive tumour cells were highly active in the adhesion of cultured cells to MVECs,

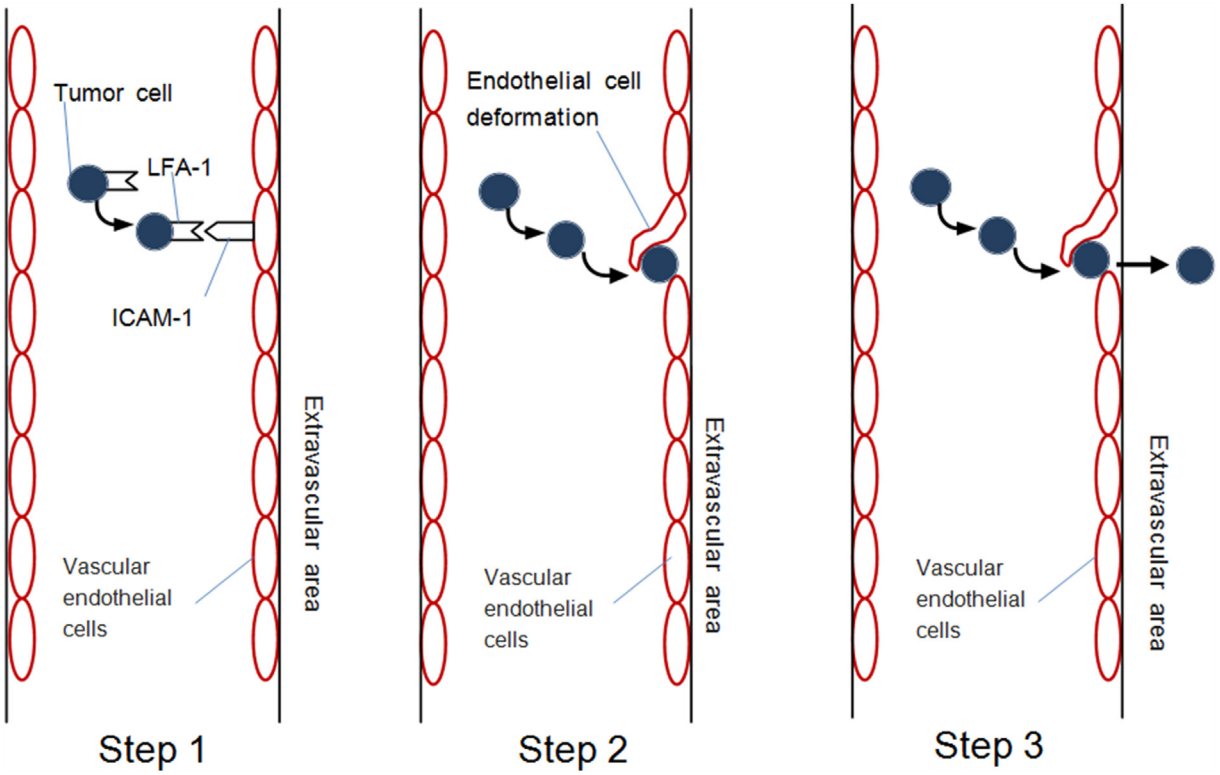

Fig. 5 The tumour cell metastasis pathway. Step 1: The LFA-1 on tumour cells binds to ICAM-1 on vascular endothelial cells. Step 2: The tumour cells exchange positions with the deformed vascular endothelial cells. Step 3: The tumour cells migrate to the extracellular matrix and pass through the space of vascular endothelial cells.

which quickly adhered to endothelial cells and constantly destroyed vascular endothelial cells (Fig. 4C–E), resulting in tumour cell metastasis. Thus, EBV is a promoter of tumour cell metastasis.

In the present study the static binding state and fast dynamic binding state of lymphoma cells with vascular endothelial cells were observed. It is speculated that tumour cells have the characteristics of both tumour dormancy and tumour invasion. The reason may be the regulation by tumour stem cells, because tumour stem cells can self-renew and promote tumour metastasis. Further explorations are needed to determine whether EBV can affect tumour stem cells.

The qualitative analysis revealed that the adhesion ability of EBV-positive cells was higher than that of EBV-negative cells. When LMP-1 appeared, this aggravated cell deterioration and metastasis. The quantitative analysis revealed that the adhesion rate of TVECs was 50% to EBV-positive cells and 5% to EBV-negative cells ($p<0.01$, Fig. 3E). The difference in adhesion rate was statistically significant. The adhesion rate of MVECs was 75% to TC-1 cells and 5% to SD-1 cells ($p<0.01$, Fig. 4B). This revealed that EBV-positive cells are more prone to cell adhesion. The difference in adhesion rate between EBV-positive and EBV-negative cells was highly statistically significant. These results reveal that the higher the adhesion rate of EBV-positive cells, the higher the metastasis rate of tumour cells. This suggests that the rate of tumour cell metastasis is directly proportional to the degree of EBV infection.

From these experimental data, the following explanations can be considered: (1) EBV stimulates tumour tissues to produce various cytokines,[19] enhances LFA-1 activity on tumour cells, and accordingly strengthens the binding with ICAM-1 on vascular endothelial cells; (2) tumour cells can degrade the basement membrane or ECM through their own proteolytic enzymes;[20] (3) tumour cells can be transferred to other tissues through the channels produced by protein degradation, such as the gastrointestinal region.[21]

In addition, it was speculated that the NF-κB signalling pathway mediates the dissemination of lymphoma cells. Hence, it can be noted that these signal transduction pathways play an important role as a bridge.

The bridge function of the NF-κB signalling pathway in cell metastasis

There is a high expression of NF-κB in lymphoma and many kinds of tumours,[22] which is continuously activated. Hence, it is the premise and basis of the anti-apoptosis effect of tumour cells.

The activation of the NF-κB signalling pathway in B lymphocyte mainly depends on the CBM complex (composed of CARD11, BCL10, MALT1 and other proteins). In approximately 10% of activated B-cell-like diffuse large B-cell lymphoma (ABC-DLBCL), the spiral domain of CARD11 mutates. This enables CARD11 to continuously activate the independence of upstream signal molecules. This results in the activation of the downstream NF-κB signalling pathway, in order to help cells escape apoptosis. Most B-cell lymphoma subtypes can maintain their own survival through this signalling pathway.[23]

After EBV infects the human body, there is a high expression level of LMP-1 in tumour cells when the anti-

tumour gene regulation is out of balance. At present, LMP-1 is defined as an oncogene,[24] and has been found in various malignant tumour cells. LMP-1 directly binds to tumour necrosis factor receptor associated factor 2 (TRAF2) through CTAR1 in the C-terminal, which activates NF-κB inducing kinase (NIK),[25,26] activates IKB regulatory kinases (IKK), and phosphorylates IKB, resulting in the separation and hydrolysis of IKB and NF-κB. In addition, NF-κB is transferred to the nucleus to play the role of a transcription factor.[27]

In addition, CTAR2 indirectly binds with TRAF2 through the action of tumour necrosis factor associated dead domain (TRADD) and activates NF-κB, while NF-κB further regulates the expression of many genes that contain κB and adhesion molecules, in order to upregulate anti-apoptotic gene products, such as Bcl-2 and A20, and upregulates cytokines such as IL-6 and IL-8, cell surface antigens such as CD54 (ICAM-1) and CD40, and angiogenesis factors such as COX2 and vascular endothelial growth factor (VEGF).[28] Furthermore, this also regulates downstream genes, such as epidermal growth factor receptor (EGFR) and p53.[29] Thus, a cell signal transduction pathway is formed, resulting in cell activation and metastasis.

Davis *et al.*[30] proposed that the chronic activation of the B cell receptor (BCR) itself can also lead to the continuous activation of the NF-κB survival promoting signal. These knock out any of the heavy chain, light chain, Igα and Igβ of immunoglobulin, which would lead to the death of the ABC-DLBCL cell line. However, the fundamental cause of spontaneous chronic activation remains unknown.

Exosomes form a microenvironment before tumour cell metastasis

When we detected the appearance of vesicles outside the tumour cell membrane, we realised that cells could not only communicate with each other through direct contact with transfer signalling molecules, but also change the tumour microenvironment by releasing extracellular vesicles, especially exosomes.[31]

These phenomena suggest that EBV stimulates tumour cells to secrete exosomes.[32] The overexpression of exosomes may be a pathological state. Jung *et al.*[33] reported that the growth and invasiveness of tumour cells increased in mice, and the reason may be the adhesion of tumour-derived exosomes to tumour cells and immune cells. Exosomes play an important role in the adhesion of tumour cells to vascular endothelial cells. In recent years, it has been reported that exosomes are closely correlated to intercellular signalling transmission, tumour development and immune escape.[34,35] Marleau *et al.*[36] used *in vitro* blood filtration therapy to eliminate the exosomes secreted by tumour cells, and the purpose of inhibiting cell metastasis was achieved.

In the detection of exosomes, we selected a electron microscope with high accuracy, and confirmed that there were single-membraned vesicles outside the tumour cell membrane (Fig. 2C), which were the exosomes produced by tumour cells. Exosomes are mainly composed of proteins, lipids and nucleic acids. There are two categories of proteins. The first category is common proteins, which include cytosolic proteins, proteins involved in intracellular signal transduction, various metabolic enzymes, heat shock proteins, and tetratransmembrane proteins.[37] The second category is specific proteins, which include exosomes secreted by antigen-presenting cells, B lymphocytes and dendritic cells, which are rich in important immune molecules, such as MHC-1, MHC-2, heat-shock proteins HSP70−90, costimulatory molecules and specific antigen peptides. These can transport the tumour antigen signal to T lymphocytes, make effective T cells mature, and initiate immune response.[38] Therefore, we believe EBV may stimulate exosomes secreted by B cells. It can stimulate the cloning and proliferation of antigen-specific CD4-positive T cells.[39]

When exosomes secreted by tumour cells were found, we speculated on the possibility of formation of the microenvironment before the metastasis of tumour cells.[40] This creates inevitable favourable conditions for the combination of LFA-1 from tumour cells with ICAM-1 from vascular endothelial cells and the cell metastasis.

Furthermore, due to EBV infection, the structure and function of vascular endothelial cells were abnormal, causing a change in the receptor-ligand binding reaction of cells. This also plays an important role in the metastasis of tumour cells.

Competitive inhibition becomes the breakthrough point to prevent tumour cell metastasis

Adhesion molecules induce the adhesion of cell-to-cell or cell-to-matrix through the form of receptor-ligand binding. These have the following functions: (1) lymphocyte homing; (2) involvement in cell recognition, cell activation and signal transduction, auxiliary receptor and co-stimulation, or inhibition of signals in immune cell recognition; (3) help white blood cells adhere to vascular endothelial cells during inflammation; (4) promote cell proliferation and differentiation; (5) participate in cell extension and migration; (6) molecular basis of a series of important physiological and pathological processes, such as immune response, inflammation, coagulation, tumour metastasis and wound healing.[41]

The binding between the receptor and ligand results in activation of the receptor and generation of the basic steps for subsequent signalling of receptor activation. When the receptor binds to the ligand, it has (1) specificity, (2) high affinity binding, (3) saturation, and (4) reversibility.[42]

In the present study, MVECs were first added with the anti-ICAM-1 antibody, and subsequently added with lymphoma cells. After one hour, no cell adhesion was observed (Fig. 4F). This result shows that the antigen on MVECs that bind with its antibody have no stress response to tumour cells. This is competitive inhibition, and in this case, LFA-1 is rejected by ICAM-1.

LFA-1 is in the bending conformation in the resting state, which transforms into an extended conformation when activated, and the affinity of receptor-ligand reaches the highest point under this condition. However, in the absence of ICAM-1 response, LFA-1 response compliance decreases, which may also be a reason for it not binding to ICAM-1.

The binding between ligand and receptor is not mediated by the covalent bond, but mainly depends on the ion bond, hydrogen bond, van Edward force and hydrophobic interaction.[43] Hence, it can be quickly reversed. This reversibility is conducive to rapid release of the signal, preventing the ligand from always being in the activated state. According to this characteristic, determining how to deactivate LFA-1 is a way to stop the cell metastasis.

Since the number of ligand molecules in cells is limited, if the concentration of the receptor molecule is increased, the ligand in cells is occupied by the receptor. However, when the receptor is in the saturated state, even increasing the concentration of the receptor does not increase the binding between the two. Therefore, when the number of receptors on vascular endothelial cells is limited and saturated, these can reject the re-adhesion of tumour cells.

After the first antibody-antigen reaction of ICAM-1, the activity of the receptor decreases or the structure of the receptor changes, and this causes the ICAM-1 on the vascular endothelial cell membrane to enter the refractory period (inactive state). Therefore, ICAM-1 does not bind to LFA-1 on the tumour cell membrane, and tumour cell metastasis is stopped. We consider that this is competitive inhibition.

Thus, it is suggested that the metastasis of tumour cells is caused by the interaction between tumour cells and target cells. This process also provides space for the intervention of cells, because if any stage of the process is destroyed, this can prevent the adhesion and metastasis of tumour cells.

From this, we are encouraged that the antibody-antigen reaction can be used to induce competitive inhibition, and destroy receptor-ligand binding, which can accordingly prevent the metastasis of tumour cells. This has clinical reference value for tumour immunotherapy.

CONCLUSION

Through the present study, we consider that EBV is an activating factor of adhesion molecules, and a promoter of tumour cell metastasis. The adhesion of tumour cells is prior to that of normal cells, and the rate of tumour cell metastasis is directly proportional to the degree of EBV infection. LMP-1 is an important factor that activates the cell signal transduction pathway. Exosomes form a microenvironment before tumour cell metastasis, which is conducive to cell metastasis. We obtained the experimental idea of receptor-ligand binding reaction through the cell's antigen-antibody reaction, and confirmed that the induction of competitive inhibition is a breakthrough point to prevent tumour cell metastasis.

Therefore, (1) inducing competitive inhibition, (2) inhibiting the secretion of exosomes, and (3) preventing the production of LMP-1 and blocking the NF-κB cell signalling pathway, are methods of reference value for tumour immunotherapy.

Acknowledgements: We would like to thank The Institute of Medical Science, The University of Tokyo, for kindly providing the SCID mice lymphoma cell line, and The Institute of Gerontology, Nippon Medical School, for kindly providing the MVEC cell line.

Conflicts of interest and sources of funding: This work was supported by the National Natural Science Foundation of China (81371672). The authors state that there are no conflicts of interest to disclose.

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