行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：☑個別型計畫　　□整合型計畫
計畫編號：NSC　90－2314－B－002－410－
執行期間：　90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：楊榮森 臺大醫學院 骨科
共同主持人：劉興華 臺大醫學院 毒理學所
計畫參與人員：黃滄海 臺大醫學院 骨科
　　　　　　　湯智昕 臺大醫學院 藥理所

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
☑出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

執行單位：臺大醫學院 骨科

中　華　民　國　　91 年　9　月　17　日

行政院國家科學委員會專題研究計畫成果報告

骨骼腫瘤血管新生因子之分析(3/3)

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一、中文摘要

骨骼是乳癌細胞最容易發生轉移的器官之一。一些動物模式的建立，為的是探討乳癌轉移至骨骼的機轉。在這些研究模式當中，局部將癌細胞直接植入骨髓腔是一種較為容易執行的實驗技術，並且所生成的腫瘤在 X 光片上也會有如同骨轉移病人的骨損壞現象。

本研究針對過去類似的動物模式簡化與修飾，使得癌細胞局部植入裸鼠骨髓腔的技術更易似施。在植入 bone-seeking type 的 MDA-231 乳癌細胞 4-5 週後，腫瘤便自脛骨骨髓腔長成，並且在 X 光片上可看見明顯的骨損壞現象。

經過活體篩選後的 bone-seeking MDA-231 細胞不同於 parent 型的 MDA-231 細胞，可直接促使破骨細胞先驅細胞生長破骨細胞，並可利用所得組織進一步研究相關生理行為。此乃是本研究的另一發現，值得進一步的研究。

關鍵詞：骨癌、乳癌、鼠科動物模式、破骨細胞、骨轉移

Abstract

Bone is one of the most preferential target organs of breast cancer metastasis. Several animal models were developed for further clarifying the bone metastasis pathway in breast cancer patients. Direct inject cancer cells into bone marrow cavity is an easier technique and also produces similar results to the radiographic photo from the patients with bone metastasis.

In the present study, we established a simpler and a more accurate local injection nude mice model as compared to the ones described previously. Four to five weeks after bone-seeking type MDA-231 breast cancer cells (MDA-231/Bone) were transplanted, tumors grew up from the tibiae and obviously osteolytic lesions were shown on the radiographic photo.

Different from parent MDA-231 cells, cloned bone-seeking type MDA-231 showed the ability to promote the osteoclast formation from stromal cells free hematopoietic cells (osteoclast precursors). Further studies would be valuable in clarifying the interaction between bone-seeking MDA-231 cells and osteoclast precursors.

Keywords: bone cancer, breast cancer, murine model, osteoclast, bone metastasis

Introduction

Ninety percent breast cancer patients dying with osteolytic bone metastasis (Walther, 1948). The mechanism of breast cancer cell metastases to bone remains unclear. Several animal models were developed for further clarifying the bone metastasis pathway in breast cancer patients.

Left heart ventricular inoculation technique of cancer cells into nude mice was a generally used model (Sasaki et al., 1995; Yoneda et al., 1997; Yoneda et al., 2000; Winding et al., 2000; Mancino et al., 2001; Peyruchaud et al., 2001; Pecheur et al., 2002). However, heart ventricular inoculation is usually technique-demanding and cannot easily make sure if the cancer cell is successfully injected into systematic circulation.

Direct inject cancer cell into bone marrow cavity is a more easily technique to perform and also produced similar results to the radiographic photo from the patients with bone metastasis (Price, 1996). In addition, local transplantation of cancer cell will help to investigate the direct

reaction between cancer cell and the possible metastasis site. Previously, several models of cancer cell transplantation into bone marrow cavity have been set up. However, complicated operation skill of Ingall's model (1964) and growth plate related damage caused by drilling needle (Berlin et al., 1993) made the experiment difficult. In the present study, we modified the injection needle, prevented the growth plate damages and successfully established an easier model for further related study.

Materials snd Methods

In vivo study

Animals: Female BALB/c-nu/nu mice (3 weeks olds) were purchased from National Laboratory Animal Breeding and Research Center of Taiwan. Transplantation of cancer cells was processed as the animals were 4-5 weeks old.

Cell culture: Human breast cancer cell line MDA-231/Parent purchased from American Type Culture Collection (Manassas, VA) and bone-seeking type MDA-231 cells (MDA-231/ Bone) cloned by our laboratory was used for present study. Cancer cell lines were cultured in Dulbecco's modified Eagle's Minimal Essential Medium (DMEM: Invitrogen, Carlsbad, California) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, California) and 1% penicillin-streptomycin solution (Invitrogen, Carlsbad, California) in an incubator with humidified atmosphere of 5% CO_2 in air. At confluence, breast cancer cells were fed with fresh culture medium 24 h before intratibial injection. Cancer cells were harvested with trypsine-EDTA (Invitrogen, Carlsbad, California), and suspended in PBS. Before intratibial injection, the cancer cells were kept under 4°C.

Intratibial Injection of MDA-231/Parent and clone of MDA-231/Bone Cells in Nude Mice: Before intratibial injection of breast cancer cell, animals were deeply anesthetized using trichloroacetaldehyde monohydrate (0.4mg/g body weight) (KANTO Chemical Co., INC., Tokyo, Japan). Intratibial cancer cells were injected using a 30 guaze needle with a polyethylene tubing (Recorder No. 427401, Becton Dickinson) fit around the needle (Fig 1). This designation is used for making sure the depth (1.5mm) of needle into the proximal tibia and for preventing the cell suspension spilling from the injection site. A 0.3ml cell suspension contained 1×10^5 cells was slowly injected into the tibial marrow cavity of 4-5 weeks old mice.

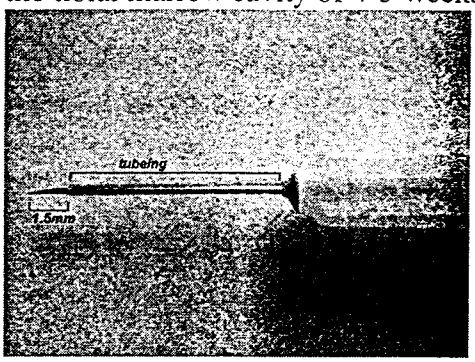

Figure 1 Needle with a polyethylene tubing fit around it

After 4-5 weeks intratibial injection of MDA-231, a visible spherical tumor grew up from the proximal tibia. In the present study, we harvested this bone-seeking tumor, wash it in DMEM, cut it to smaller pieces and directly cultured the tissue piece in the DMEM. The tumor cells will migrate from the tissue to the plastic 175cm^2 flask. We maintained this bone-seeking tumor cell MDA-231/Bone in culture for six passages for further intratibial transplantation and coculture experiments.

Radiographs: For making sure of bone osteolysis, radiographs were taken by a soft X-ray generating unit (Young-kid Enterprise Co., Ltd., Taipei, TAIWAN). At the end of the experiment, animals were anesthetized deeply as before, laid down in a prone position on a Korda Scientific Imaging film (13×18 cm;), and X-ray exposure was perform at a 45kVp for 5 seconds.

H-E stain: In the end of the experiment, animals were anesthetized deeply and resected the hindlimbs immediately. For further histological analysis, the hindlimbs were fixed using 4% paraformadehyde in PBS for more than 72 h, decalcified in a 14% EDTA solution for 14 days, dehydrated in increasing concentration ethanol and embedded in paraffin. The serial histological sections were cut longitudinally and stained with Mayer's hematoxylin-eosin solution.

In vitro study

Hematopoietic cells as osteoclast precursors: Primary bone marrow cells were collected from an S.D. rat of 45-60 days old. Bone marrow cells were washed out from the bone marrow cavity of

tibiae and femurs. After a 24-hour incubation, nonadherent hematopoietic cells were collected and used as osteoclast precursors.

Coculture of cancer cell and hematopoetic cells: In the present study, three doses of MDA-231/Parent and MDA-231/Bone (2×10^3, 1×10^4, 5×10^4 cells each) were cultured in 24-well plates with hematopoetic cell (1×10^6/well). Cultures were maintained for 9 days in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution. After 9 days culture, cells were fixed and stained with TRAP (Sigma Diagnostics Inc., Louis, MO, USA). TRAP-positive and multi-nuclei (over 3 nuclei) were counted as osteoclast.

Results

After 4-5 weeks transplantation of bone-seeking breast cancer cells (MDA-231/ Bone) into tibia of nude mice, 8 of 10 tibiae showed solid tumors from the bone marrow cavity and an osteolytic lesion was shown as compared to the tibiae of control group (Fig 2).

We harvested the tumor tissues and cultured it (MDA-231/Bone) for further *in vitro* study. Coculture of hematopoietic cells with three cell doses of MDA-231/Bone showed a dose dependent formation of osteoclast number (Fig 3A) but not with MDA-231/Parent (Fig 3B). Figure 4 showed the typical osteoclast formation from MDA-231/Bone cells and hematopoietic cells coculture. In addition, MDA-231/Bone cells also showed a different morphology as compared to MDA-231/Parent cells (Fig 5).

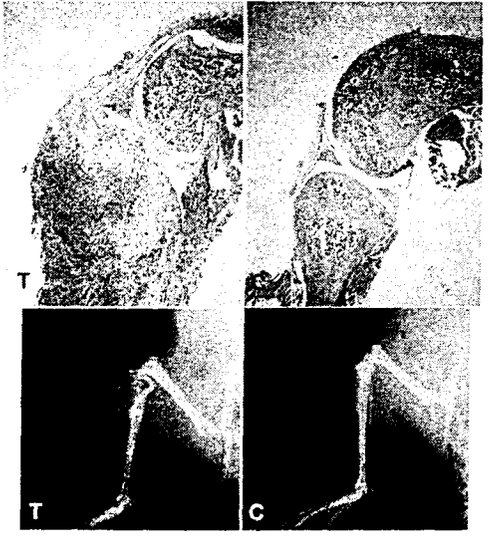

Figure 2: MDA-231/Bone cells transplanted into bone marrow cavity and an solid tumor was grew up after 4-5 weeks

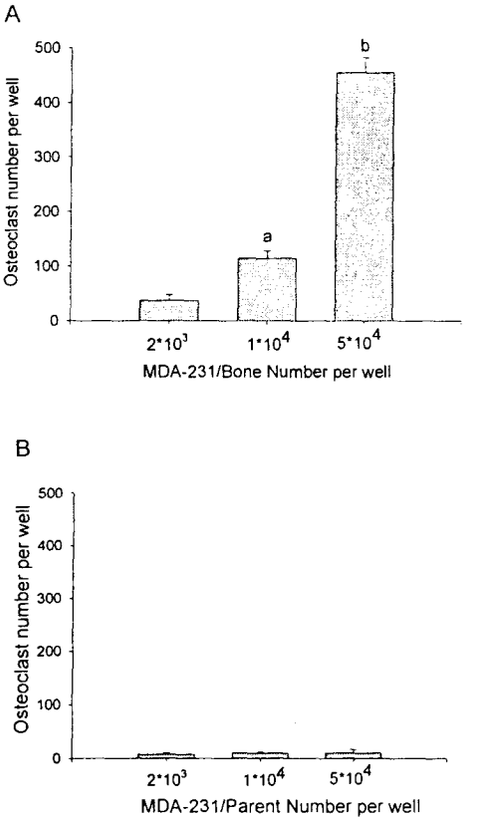

Figure 3: Three doses of MDA-231/Bone (A) and MDA-231/Parent (B) cocultured with hematopoetic cell ($1*10^6$/well) (key: a, $1*10^4 > 2*10^3$; b, $5*10^4 > 1*10^4, 2*10^3$, $p< 0.001$)

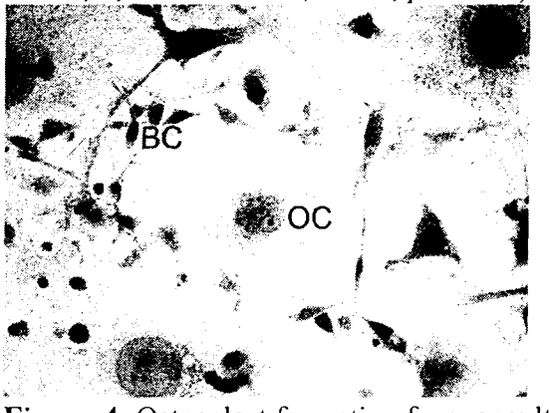

Figure 4: Osteoclast formation from coculture of MDA-231/Bone with hematopoetic cell (Key: OC, osteoclast; BC, breast cancer cell)

Disscussion

In the present study, we successfully established a model using local transplantation of cancer cell. Although, this model could not mimic the metastatsis of cancer cell as well as the model of heart ventricular inoculation, this model provides us an another way to investigate the directly interaction between cancer cell and the possible metastasis site.

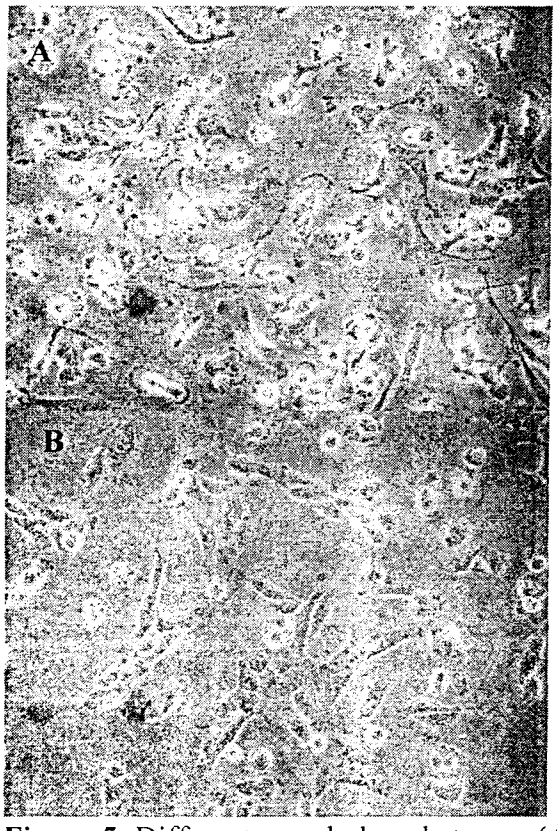

Figure 5: Different morphology between (A) MDA-231/Bone and (B) MDA-231/Parent

During the process of injection, cancer cell suspension easily spills out from the injection site. In addition, the way of intratibial injection under growth plate cannot easily make sure the depth of needle.site. Therefore, it is not unusual that the needle is over input into the tibia and the orthotopic injection site is missed. We modified the needle with an polyethylene tubing fit around it, which is helpful in making sure the 1.5 mm depth of needle input and successfully prevented the defects described previously. As compared to the model of Ingall (1964), we did not need a complicated technique but just a simple injection skill. Injection under the growth plate made our model avoiding damage to the tibial growth plate as Berlin's method might happen (1993).

In the coculture experiment, the cloned bone-seeking tumor cell MDA-231/Bone seemed to directly interact with hematopoietic cell and promoted the formation of osteoclast with a dose response. However, MDA-231/Parent cell did not promote the generation of osteoclast as coculture with hematopoietic cell. As previous study (Mancino et al., 2001) has referred, human breast cancer line MDA-231 might stimulate the osteoclast formation through upregulating the receptor activator of the NF-κB ligand (RANKL) of stromal cell. However, the bone-seeking MDA-231/bone could directly promote the stromal cell free hematopoietic cells differentiating to osteoclasts. Further studies should be processed to clarified the mechanism of MDA-231/Bone cell in promoting the osteoclast formation.

In conclusion, local transplantation technique of cancer cells, which was established in present study was easily to perform and would be helpful for further studies in investigating the interaction between the cancer cells and their high frequency metastasis site.

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國科會補助專家學者出席國際會議報告

90 年 10 月 20 日

報告人姓名	楊 榮 森	服務機關名稱※及職稱	台大醫學院 骨科 教 授
會議期間及地點	90年10月10日至90年10月12日 英國 伯明罕	本部核定補助文號	[redacted]
會議名稱	(中文)第十一屆國際肢體保全學會研討會議 (英文) 11th International Symposium on Limb Salvage		
發表論文題目	(中文)人類乳癌轉移骨骼病灶的 bFGF, PDGF 和 VEGF 的表現 (英文) The Expression of bFGF, PDGF and VEGF of Human Metastatic Breast Cancer Tissues		

報告內容應包括下列各項：

一、參家會議經過

二、與會心得

三、建議

四、攜回資料名稱及內容

五、其　　他

※服務機關若係大專院校，請註明科系名稱。

※報告內容請另以稿紙書寫工整，俾本部輯印成冊送各相關單位參考應用。

第十一屆國際肢體保全學會研討會議

楊榮森
臺大醫學院骨科

(一)參加會議過程

第十一屆國際肢體保全學會研討會議的會議日期自 2001 年 10 月 10 日至 10 月 12 日，共計三天，會議地點在英國伯明罕市的國際會議中心 (International Convention Center, Birmingham, UK)舉行，本次會議由國際肢體保全學會主辦，大會的會長爲英國的 Simon Carter，他是當今的骨腫瘤著名大師，對於骨骼腫瘤肢體保全手術具有很傑出的貢獻。本次會議乃是國際性儲議，因此與會者來自世界各地的骨腫瘤醫師同好，包括美國，歐洲各國，如英國、法國、德國、義大利、奧國等，巴西，亞洲各國，如臺灣、日本、韓國、澳洲、新加坡、印度、泰國等，本次會議原先預料會比先前數屆更爲盛大，雖然由於在 2001 年 10 月 7 日美國與阿富汗的戰爭引爆，使許多會員未能與會，但仍有約近二百五十名骨腫瘤專家與會，當真盛況空前。

本次會議爲令與會者能夠全程參與，會議型式採在同一演講堂的方式進行，10 月 10 日會議前，大會單位並邀請伯明罕市長主持開幕儀式，尊行英國古禮，相當特別。然後即時進行各項研討主題的演講。全程會議共有 兩個特別演講，討論有關生物力學及組織工程的重要成就，其中更討論有關化學治療對骨骼的癒合及生長影響。此外，本次大會共有 15 個主題論文討論，其中許多都最近發展的新熱門主題。此外，大會也安排一些特殊病例請專家提供手術意見。大會另外安排在早上及中午時段，由不同學者同時進行溝通及海報的觀賞討論，內容項目與前述相似，讓與會者和作者可直接溝通交談，效果相當良好。大會的主題研討會安排在一個大會場舉行，會場設備新穎，容量很大，足可容納所有會員，會場視聽效果很好，可惜仍有些幻燈片較爲暗淡，是爲美中不足處。

在本年度第十一屆國際肢體保全學會研討會議中，共有 268 篇論文發表，包括 107 篇口頭報告論文與 161 篇壁報論文，這些論文都是骨腫瘤的最新研究成果報告，但可惜因戰爭關係，有些學者並未參與。今年的口頭論文報告中，內容包括良性骨腫瘤，放射線學檢查，骨盆腫瘤，脊椎腫瘤，腫瘤病患的功能，上肢骨腫瘤，組織學，病患資料收集及追蹤，轉移疾病，軟組織腫瘤，新式手術技術，孩童骨腫瘤，骨腫瘤再度手術及併發症，下肢骨腫瘤，體外處理骨骼的重建等，這些都提出現今對於骨腫瘤肢體重建手術的新知見解，相當令人關心注意。

第十一屆國際肢體保全學會研討會議的壁報論文發展仍爲本年度研討會的重點工作之一，在此聚集世界各地精英學者發表其豐碩研究成果，以便與會學者共同討論及分享研究心得，提供各位學者多次觀摩機會，今年度壁報論文的層面非常廣且深入，經由大會精心安排與會者發表研究成果，內容包括有關骨腫瘤的各方面，從基礎到臨床，從診斷到治療，從動物實驗到人體研究，一應

齊全，由內容可知，大家開始重視骨腫瘤的基因研究，本年度亦有不少論文對此進行深入，完整且簡要的討論。第十一屆國際肢體保全學會研討會議壁報論文的主題經由大會精心安排，便利與會學者作重點式參觀。作者今年參與會議的壁報論文題目爲『人類乳癌轉移骨骼病灶的 bFGF, PDGF 和 VEGF 的表現(The Expression of bFGF, PDGF and VEGF of Human Metastatic Breast Cancer Tissues)』，由於乳瘤惡性度很高，生長速度極快，且常常會發生到骨骼部位的轉移病灶，這些轉移性骨腫瘤都是血管豐富的腫瘤，鑑於腫瘤生長與血管新生之間關係十分密切，腫瘤本身可能合成一些促進血管新生的因子，且血管內皮細胞可能也會分泌一些促進腫瘤生長因子；因此本研究利用利用人體骨骼轉移性乳癌組織進行一系列研究，探討各種血管新生因子與骨骼腫瘤的關係，包括纖維母細胞生長因子(basic Fibroblast growth factor, bFGF)，血小板源性生長因子(Platelet-derived growth factor, PDGF)，和血管內皮細胞生長因子(Vascular endothelial growth factor, VEGF)等，並研究其與腫組織內血管分佈及治療情形的關係。本研究共取得 24 例乳癌轉移骨骼的病患腫瘤組織進行研究，包括(1)免疫組織化學染色方法(探討骨肉瘤組織及乳癌的轉移性骨腫瘤中的 bFGF, PDGF,或 VEGF 之分佈及表現情形，並評估腫瘤組織內的血管密度)，(2) 利用西方墨點法(Western blot)方法和免疫吸附定量法(Immunosorbent assay)測定腫瘤組織內血管新生因子含量，並與腫瘤種類作比較。結果發現乳癌轉移骨骼腫瘤組織中，共有 18 例出現 bFGF 陽性反應(75%)，16 例呈 VEGF 陽性反應(67%)，20 例則出現 PDGF 陽性反應(83%)，其陽性反應也與腫瘤組織內的血管密度呈正相關。西方墨點法和免疫吸附定量法的研究結果顯示，15 例乳癌轉移骨骼腫瘤組織中共有 9 例出現 bFGF 陽性反應(60%)，11 例出現 PDGF 陽性反應(73%)，9 例出現 VEGF 陽性反應(60%)，經由本研究得以進一步明瞭解骨肉瘤細胞的生物特性，並供臨床治療參考。

總言之，第十一屆國際肢體保全學會研討會議的議程安排非常完善，且由大會主辦單位所精選的的研究主題研討會議，在在顯示出當前的重要研究主流，且由於大會的會議進行順利，令與會者可以便利得到有關骨腫瘤診治的最新發展資訊，事半功倍，獲益匪淺。

(二)與會心得

第十一屆國際肢體保全學會研討會議非常圓滿成功，當然在於許多學者的參與，在大會中所接受發表的論文數目很多，而根據大會安排的特別演講及專題討論內容，可令人容易明白骨腫瘤的整體現況與未來研究發展方向，本次大會主要重點包括仍爲骨腫瘤的診斷與治療，其中包含各種新式治療的重要發展，但一些有關新式化學治療藥物的基礎研究，骨腫瘤的基因研究項目，及關於骨腫瘤病患的生活品質之探討，也漸漸受到重視，此外，大會更刻意安排一些醫學以外的領域供其他人員參與，更能顯示出本次大會重視整體性的決心。這些趨勢顯示，世界各地對骨腫瘤已漸達成共識及整合，對於未來大規模研究的脈動與方向，更值得密切注意。

本年度大會刻意安排組織工程與骨腫瘤關係的探討，這些要項更是推廣骨腫瘤診治療上的重要基石，對於在切除骨腫瘤後所造成的缺陷，需要精心的重建，且需使用很多的重建材料，因而使骨骼相關的組織工程成爲目前的熱門研究主題，許多肢體重建工作皆須借助骨骼組織重建工程的協助，目前雖然仍未能見其功效，但相信指日可待。此次大會能推廣這些要項，自當值得深恩。

在本次的學術大會中，在診斷骨腫瘤的方法中，仍注重早期診斷，早期治療。許多現代化的檢查十分重要，但應注重對病患的身體檢查。此次開會中曾有論文提及利用同位素掃瞄檢查來評估病患的化學治療成效評估，對於其治療預後的判定也很有幫助。筆者也對於相似題目做深切的研究，並在本年度中華民國骨科醫學會發表，結果發現若在化學治療後，同位素掃瞄檢查的活性不降反高，則與病患發生肺部轉移或局部再發方間具有密切關係，本論文且得到本年度骨科醫學會的論文比賽佳作。

在治療骨腫瘤方面，其中的主要治療項目乃爲肢體保全手術。由於骨腫瘍的治療方式很多，且通常會依治療個別病患而略有差異，因此在治療上需注意不同的病患情況，而做適當調整。本次會議也曾對兩例病患提出其病情，並邀請與會的著名學者，分析其所使用的治療方式建議，並將其思考過程做適當分析後，再由與會者投票選擇，最後由該病例的醫師提出其做法及病患近況分析。經由如此的討論後，即可明白許多不同地區國家的學者，由於各國的經濟及經驗不同，因而對於治療同一位病患的看法，實在具有很大的差異性。

在基礎研究上，有些學者報告有關動物模式的治療成效，也有一些是骨腫瘤細胞的研究報告，這些都是對開發新藥非常有用的模式。許多研究者對於新藥仍保持相當保守的態度，來自美國紐約的 John Healey 也提出有關骨腫瘍內血流壓力的研究報告，許多病患的骨腫瘤內部血管壓力比正常部位的血管內壓力高，這個發現的可能具體臨床意義爲何，仍值得進一步的觀察。

總之，第十一屆國際肢體保全學會研討會議實在相當成功，許多與會學者的熱烈參與，使大會的研究討論廣泛深入，大會主辦會議的熟練，值得學習，但對會議進行的時間並未良好掌控，也值得注意。

(三)建議

今年參加第十一屆國際肢體保全學會研討會議，發現許多國家仍注重新式的治療及診斷發展，多年來參與國際會議後，仍深深感到國際會議對凝聚國際重要研究資源的重要，在這些研究會議的進行中，可深切且直接切入許多重要相關領域的研究心得，甚至於可明白當前的重點研究新發展，適當調整研究方向。在與會者的成員中，歐美國家仍是主要成員，此次國人共有五位參加，而其他亞洲國家中以日本最多，韓國也很多，今年更可見到其他亞洲國家的醫師參與，可見各國對此會議重視。目前國內同好已互相溝通，希望成立連絡機構，希望來年可有更多志同道合學者相互砌磋，發表研究心得，以提高國內的骨腫瘤診治研究水平，並揚名國際。更希望能夠得到國家的補助，帶領一些年輕學者參與，增多研究人口，並使他們更早登堂入室。

Title:

The Expression of bFGF, PDGF and VEGF of Human Metastatic Breast Cancer Tissues

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Subtitle: bFGF, PDGF and VEGF in Human Metastatic Breast Cancers

Abstract

New diagnostic and therapeutic techniques have improved much the survival of patients with breast cancers. The increase of 5-year survival rate is accompanied by the increased frequency the late bone metastasis. The meatstatic bone lesions of breast cancer are highly vasculaized. Furthermore, the tumor growth is closely related to the neoangiogenesis. Therefore the relationship between the angiogenesis factor and these tumors merits further investigation. In this study we used immunohistochemistry with monoclonal antibodies against bFGF, PDGF and VEGF to evaluate the expression of bFGF, PDGF or VEGF in 24 human metastatic bone tissues specimens from breast cancer. In addition, another 15 deep-frozen bone metastatic tissues from breast cancer were obtained for Western blot analysis of the expression of bFGF, PDGF or VEGF. The results of immunohistochemical study showed the localization of the bFGF (75%), PDGF (83%) and VEGF (67%) in the metastatic bone tumors tissues. Moderate to intense staining of endothelial and perivascular muscle cells were also shown in tumor tissue specimen for bFGF, PDGF and VEGF expression. The bFGF, PDGF and VEGF staining in tumor cells was positively related to the microvessel densities. The Western blot analysis also showed the expression of bFGF (60%), VEGF (60%) and PDGF (73%)in tumor tissues. This study may help us to understand the basic biological behavior of the metastatic bone lesions from breast cancers. Further research is warranted to determine the its utility in the clinical practice, including the evaluation of treatment response.

Keywords: bFGF, PDGF, VEGF, Breast cancer, Angiogenesis factor, Immunohistochemistry, Western blot

Introduction

The advent of various diagnostic and therapeutic techniques has improved much the survival of patients with breast cancers. However, the longer survival times may be accompanied by the increase of late bone metastasis. Among these metastatic bone tumors include osteolytic bone lesions that compromise the normal strength and cause pathological fractures. The previous study demonstrated that the tumor growth is closely related to the neoangiogenesis [1-12]. Besides, the meatstatic bone lesions of breast cancer are usually highly vascular. Thus the relationship between the angiogenesis factor and these tumors merits further investigation.

Tumor cells may synthesize some kinds of angiogenesis factors to regulate the neoangiogenesis [9-13]. On the other hand, the endothelial cells can also produce some cytokines to stimulate the growth of the tumor cells. Such an investigation will shed a highlight on the biological behaviors of the metastatic breast cancers. It may help us to understand the response to therapy and its prognosis.

The previous studies showed that tumor cells could synthesize some kinds of angiogenesis factors to regulate the neoangiogenesis. On the other hand, the endothelial cells can also produce some cytokines to stimulate the growth of the tumor cells [9-22]. The common angiogenesis factors include basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) , and vascular endothelial growth factor (VEGF) [9-22]. These factors may stimulate the proliferation of endothelial cells and the formation of new vessles. Thus, we investigate the expression of angiogenesis growth factor, including bFGF, PDGF and VEGF in the metastatic bone lesions [9-13].

The antibodies to bFGF, PDGF or VEGF permit an easy and simple immunohistochemical procedure or Western blotting analysis for rapid determination of bFGF, PDGF or VEGF in tumor tissue specimens. This report details the immunolocalization of bFGF, PDGF or VEGF in 24 human metastatic bone tissues from breast cancers using monoclonal antibodies against bFGF, PDGF or VEGF. In addition, we investigated the expression of bFGF, PDGF or VEGF by Western blotting method in 15 frozen tumor specimens.

Materials and Methods

Twenty-four human metastatic bone tissues specimens from breast cancer were obtained for Immunohistochemistry of the expression of bFGF, PDGF or VEGF. These specimens were previously demineralized fixed in 10% formalin and embedded in paraffin wax. Another 15 deep-frozen bone metastatic tissues from breast cancer were obtained for Western blot analysis of the expression of bFGF, PDGF or VEGF.

Immunohistochemistry

Sections were deparaffinized in xylene and dehydrated in graded ethanol. For antigen retrieval, slides were bathed in 0.1% Protease (Sigma, St. Louis, Missouri) / PBS (pH 7.4) for 15 minutes at 37°C and then washed in PBS (pH 7.4). Sections were treated with 3% H_2O_2 for 30 minutes at room temperature to block endogenous peroxidase activity. Slides were incubated with the bFGF, PDGF or VEGF antibody (1 μg/ml, Santa Cruz Biotech, USA) for one hour at room temperature in a humidity chamber. Antibody to bFGF, PDGF or VEGF is a monoclonal antibody found to specifically react against bFGF, PDGF or VEGF. The specificity of antibody was demonstrated by Western immunoblotting, immunoprecipitation and immunohisotchemistry techniques. After primary antibody incubation, slides were washed with PBS and then incubated with Link antibody (Dako Ltd, High Wycombe, UK) for 15 min at room temperature in a humidity chamber. Then, sections were incubated with streptavidin-biotin complex (Dako Ltd, High Wycombe, UK) for 15 min at room temperature in a humidity chamber. Finally, 3-3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) solution (containing 0.024% H_2O_2 and 0.067% DAB in PBS, pH 7.4) was applied. Slides were counterstained with Harris' hematoxylin, dehydrated in graded ethanol, and coverslipped for final histologic analysis. For negative control, certain specimens were incubated with isotype-matched mouse IgG (1 μg/ml) for two hours at room temperature in place of primary antibody. All sections and controls were evaluated by three independent observers to confirm the presence of bFGF, PDGF and VEGF. The correlation between the expression of bFGF, PDGF and VEGF was then made with the microvessel density of tumor tissues. Chi-square test and Fisher exact test were used to evaluate the statistical significance which was determined to have a $p < 0.05$.

Western blot analysis

A 30-50 μg sample protein of tissue lysate was applied to electrophoresis on an 8% SDS-polyacrylamide gels for detecting the expression of bFGF, PDGF or VEGF. The samples were then electro blotted onto nitrocellulose papers. After blocking, the blots were incubatedi th anti- bFGF, PDGF or VEGF antibodies (Transduction Laboratories, USA) in PBS/Tween 20 for 1h followed by two washes in PBS/Tween 20. Then the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel, USA) for 30 min. The expression of antibody-reactive bands of bFGF, PDGF and VEGF were shown using an enhanced chemiluminescence kit (Amersham, UK). All positive controls were obtained from Transduction Laboratories (USA). Moreover, α-tubulin served as control for sample loading and integrity.

Results

Immunohistochemical study showed the localization of the bFGF, PDGF and VEGF in human metastatic breast cancer tissues with monoclonal antibodies against bFGF, PDGF and VEGF. Strong brown staining for bFGF, PDGF or VEGF was demonstrated in the cytoplasm of tumor cells (Fig. 1A, B & C). Human metastatic bone tissues from breast cancer specimens treated with mouse IgG in place of the primary antibody demonstrated no staining (Fig. 1D). Strong brown staining was demonstrable in the cytoplasm of tumor cells indicating the presence of various angiogenesis factors. The results demonstrated that 18 of 24 specimens (75%) having the presence of bFGF in tumor cells, for VEGF, 16 of 24 (67%); and PDGF, 20 of 24 (83%) (Fig 1). Moderate to intense staining of endothelial and perivascular muscle cells were also shown in tumor tissue specimen for bFGF, PDGF and VEGF expression was found. The bFGF, PDGF and VEGF staining in tumor cells was positively related to the microvessel densities.

In addition, the Western blot analysis also showed the expression of bFGF, PDGF and VEGF proteins in human metastatic breast cancer tissues (Fig. 2A). Among them 9 of 15 (60%) showed the presence of bFGF in tumor tissues, for VEGF, 9 of 15 (60%); and for PDGF, 11 of 15 (73%) (Fig 2B).

Discussion

This study demonstrated the immunolocalization of bFGF, PDGF and VEGF in tumor cells in the specimens of 24 patients with metastatic bone lesions from breast cancers. These angiogenesis growth factors were shown in both the tumor cells, as well as in the endothelial cells and perivascular muscle cells. The expression of bFGF, PDGF and VEGF in the tumor cells was associated with significantly microvessel densities. Furthermore, the expression of bFGF, PDGF and VEGF has been shown using the Western blotting technique. These results demonstrated that the metastatic vascular bone lesions expressed a high content of angiogenesis factors.

The advent of various diagnostic and therapeutic techniques has improved much the survival of patients with breast cancers. The 5-year survival rates increase is accompanied by the increase the late bone metastasis. Among these metastatic bone tumors include osteolytic bone lesions that compromise the normal strength and cause pathological fractures eventually. In addition, the metastatic lesions may be accompanied by other medical complications, including hypercalcemia, tumor emboli, etc. A proper control of osteolytic metastatic lesions may slow down the progression and extent of bone destruction, and help the control of underlying disease course [4-8]. Since tumor growth is closely related to the neoangiogenesis and meatstatic bone lesion of breast cancer are highly vascular, the relationship between the angiogenesis factor and these tumors merits further investigation.

The process of tumor metastatic cascade includes the proliferation of tumor cells, transmigration of tumor cells into circulation, adhesion of tumor cells to the endothelium, transmigration to the perivascular tissues, proliferation of tumor cells in a new site and form a new lesion [4-8]. These processes are accompanied by enzymatic digestion of the matrix as well as neoangiogenesis [9-11]. The neoangiogenesis needs an effect of enzyme to the basement membrane of small venule, followed by chemotaxis of endothelial cell and causing proliferation of these cells, budding of new vessels and forming a new vessel.

The neoangiogenesis can occur in a physiological way, such as development and growth, wound healing, regeneration of tissue or organ. On the other hand, neoangiogenesis may also occur in a pathological way, including tumor growth,

metastasis, arthritis, retinopathy, etc.[12-18]. Theoretically, the growth of tumor or metastasis is closely related to the neoangiogenesis. The newly formed vessels provide the essential nutrient and oxygen, transport the metabolic wastes, and may provide a inlet of tumor cells into circulation because of the defect of vessl wall structure [9-18].

The previous studies showed that tumor cells could synthesize some kinds of angiogenesis factors to regulate the neoangiogenesis. On the other hand, the endothelial cells can also produce some cytokines to stimulate the growth of the tumor cells [9-22]. The common angiogenesis factors include basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) , and vascular endothelial growth factor (VEGF) [9-22]. These factors may stimulate the proliferation of endothelial cells and the formation of new vessles. Thus, we investigate the expression of these angiogenesis growth factor in the metastatic bone lesions, including the surgical specimens.

VEGF, also known as vascular permeability factor (VPF), is a specific growth factors of endothelial cells [1,9-14, 19-22]. VEGF is a dmier glycoprotein. Its structure is similar to the PDGF. Various transcriptions may cause the different subtypes of VEGF, and containd 121, 165, 189, or 206 amino acids [14,19,20]. VEGF is closely to the growth of vessel endothelial cells. VEGF has been shown to present in the adult tissues or cultured cells with a complicated physiological fuction [14,22]. Since the metastatic tumor is usually highly vascular, the VEGF may play an important role in the regulation of tumor growth and metastasis. In the current study, we have demonstrated a high percentage (60-67%) of VEGF in the tumor tissues. Therefore, the clinical implication of VEGF in the control of metastasis merits a further investigation.

Fibroblast growth factor (FGF) is a family of growth factors, including at least 7 peptides containg 150-250 amino acids. bFGF, extracted from placenta, contains 157 amino acids and its MW is 17.5kDa [17,18,24,25]. The previous researches demonstrated bFGF is present in the endothelial cells, macrophages, osteoblasts, and prostate cancer cells [26,27]. The previous reports have shown its role of regulating neoangiogenesis in the adrenal glands, retina, kidney, and ovary, etc. Therefore, bFGF may be related to the reproduction, growth, development, tumor growth and metastasis, and retinopathy [9-18,25,28].

Platelet-derived growth factor (PDGF) is a thermally stable molecule, consisting od dimmer glycoprotein and having a MW of 30kDa. PDGF has been shown to be present in the platelets, macrophages, certain tumor cells including osteosarcoma and soft tissue sarcomas [25, 29-32]. PDGF has been shown to regulate the activation or secretion of proteolytic enzymes in cancer cells and may mediate the invasive and metastatic behavior of these cells [3]. The high content of PDGF in the blood clot may be related to the neoangiogenesis. Furthermore, PDGF is related to the normal growth, development, wound healing, atheroscelrosis, chronic inflammatory disease and tumor growth [2,25,30,31]. The PDGF may stimulate the proliferation of tumor cells and may also stimulate the process of neoangiogenesis.

In this study, monoclonal antibodies against bFGF, PDGF and VEGF were used to detect these proteins in 24 tumor specimens taken before administration of chemotherapy. The samples were chosen to provide the maximum percentage of viable cells, allowing an accurate assessment of the tumor's bFGF, PDGF and VEGF-producing capability. The results were graded according to the percentage of cells in the tissue that stained positive for bFGF, PDGF and VEGF. Further study about the chemotherapy on the expression of the angiogenesis factors may provide a possible tool for the evaluation of the effect of chemotherapy.

In conclusion, bFGF, PDGF and VEGF expression was demonstrated in the metastatic bone tumor tissues from breast cancer. The presence of this protein prepared from paraffin specimen was associated with a higher content of microvessels. Such an investigation will shed a highlight on the biological behaviors, response to therapy and its prognosis of metastatic breast cancers.However, a sample size precluded any conclusion regarding ultimate prognosis thus requiring the need for a further large scale investigation.

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Figure Legends:

Fig. 1 Immunohistochemical localization of the bFGF, PDGF and VEGF in human metastatic breast cancer tissues. Strong brown staining for bFGF, PDGF, VEGF was demonstrated in the cytoplasm of tumor cells (A, B & C). The negative controls were not stained (D).

Fig. 2 Western blot analysis of bFGF, PDGF and VEGF proteins in h m an metastatic breast cancer tissues. These proteins were separated by SDS-PAGE and analyzed by immunoblotting techniques as described in Materials and Methods..