3．研究成果の刊行に関する一覧表

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４．研究成果の刊行物・別刷

MEDICAL TOPICS 第14回

γ-グルタミルトランスペプチダーゼ（γ-GTP/GGT）の骨吸収亢進作用

(Osteoclast forming activity of γ-Glutamyl transpeptidase)

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Key words：γ-GTP，破骨細胞，骨吸収

はじめに

肝機能マーカーであるγ-GTPは広く市井に行き渡った検査用語の1つである。酒呑みのオジサンたちはその数値に一喜一憂しながらも呑んでいる。むろんγ-Glutamyl transpeptidase[1]のことである。国際標準で表記するならGGTとなるが，ご多分にもれず国際標準など通用しない。わが国ではγ-GTPはこれからもγ-GTPなのだろうが，ここではGGTと表記する。GGTは，一般検査においてはアルカリフォスファターゼ（ALP）などとともに「胆道系酵素」として黄疸の鑑別，肝・胆道系疾患の診断や経過観察に用いられている。GGTは肝細胞の毛細胆管の膜表面に発現し，胆汁うっ滞，アルコール性肝障害，薬剤性肝障害が起こると膜から離れ血中に入るのでその数値が高くなるからである。つまり，血中GGTは主に肝臓由来ということになる。だから肝機能マーカーなのであろう。ある種の癌では発現が高く，その種の腫瘍マーカーになることもある。この酵素が最も大量に発現している臓器は腎臓（近位尿細管）である。GGTは細胞外においてグルタチオンなどのγ-Glutamyl基をほかのタンパクに転移するという重要な役割を担っている。この過程の中にはグルタチオンの分解に依存する細胞内へのシステイン補充という役割が組み込まれている[2]。だから，GGTの欠損が起こるとシステイン欠乏になる。GGT遺伝子欠損マウスでは白内障，短寿命などの老化現象的な症状がみられる[3]。これはGGTによる細胞内へのシステインの供給が途絶え強力な抗酸化物質であるグルタチオンの産生システムが働かず，酸化ストレスに対する恒常性維持機構が破綻したためと考えられている。骨の代謝も不調になり骨粗鬆症様を呈する[4]。これらの症状はシステインの投与で回復するのだから酵素活性に関連した現象と思われる。ところが，本稿で紹介するGGTの作用はこうした酵素活性とは無関係のようである。

骨吸収因子GGTの単離

体の骨は吸収と形成を繰り返している。これを骨のリモデリングという。3年もあればすべての骨は新しくなるらしい。吸収を担当している破骨細胞はマクロファージ系の多核巨細胞である。その証拠に，この細胞の分化にはマクロファージと同じ成長因子（CSF-1/M-CSF）が必須である[5]。CSF-1で誘導された破骨細胞前駆細胞は，骨芽細胞から分泌される破骨細胞分化因子（RANKL/TRELANCE/POGL）によって最終分化を遂げる[6]。CSF-1とRANKLの産生はインターロイキン（IL）-1，IL-6，IL-17，プロスタグランジンE_2，ビタミンD_3などの刺激で上昇する[7]。ほとんどが炎症性の因子だが，骨の分野ではこれらを骨吸収因子と呼ぶ。骨吸収因子は女性ホルモンの欠乏，

リウマチや歯周炎などの炎症で発現が亢進する。

癌や炎症組織からはさまざまなサイトカイン，化学物質が産生される。骨に発生した癌や炎症組織にはまだまだ未知の骨吸収因子の発現があるのではないか。マウスで骨に転移すると顕著な骨破壊を惹起するTリンパ腫細胞(BW5147細胞)の発現遺伝子群を調べてみた。これらの細胞を増殖させて細胞からRNAを取り出す。それらを長さ別にいくつかに分けてアフリカツメガエルの卵の中に入れて培養する。培養液には卵で合成されたTリンパ腫細胞由来のタンパクも分泌されている。それを骨髄細胞の培養に入れてやる。ある長さのRNAフラクションを注入した卵の培養液に強い破骨細胞誘導活性が示されたので，そこからcDNAライブラリーを構築し，再びcRNAにして卵に入れ，同じことを繰り返す(**図1**)。書くと簡単であるが実は大変な作業である。このフラクションからのクローンだけでも63万個もできた。こうした作業を繰り返し，破骨細胞形成活性をもつ遺伝子を絞り込む。単離したものを順にその塩基配列を調べるのだが，すべてが既知のものならこの仕事はそれでおしまいである。ところが，運よく既知の骨吸収因子のリストにないものが含まれていた。

GGTの破骨細胞形成能

塩基配列のデーターベース検索でGGTがヒットしたときは何かの間違いだと思った。GGT自体は未知のものどころか，酔っ払いのオジサンだって知っている。しかし，骨吸収因子としては新規のものである。GGTに骨吸収活性などあるはずがない。ほとんどの人はそう思うらしい。そこで，ラットの腎臓から精製したGGT(阪大・谷口直之教授提供)を骨髄培養に直接加えてみた。既知の強力な骨吸収因子ほどではないが，この系でも確かに多核の破骨細胞様細胞の誘導が確認された(**図2**)。これらの細胞を象牙板の上で培養すると象牙質を吸収することも確かめた。さらに，

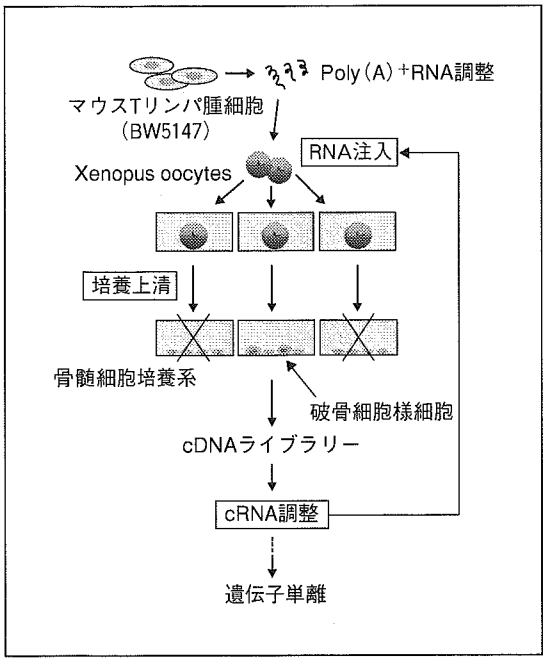

図1．発現クローニング法の略図
マウスTリンパ腫細胞から取り出したRNAをアフリカツメガエル卵に注入してTリンパ腫細胞由来のタンパク合成を代替させる方法で破骨細胞誘導因子を取り出した。

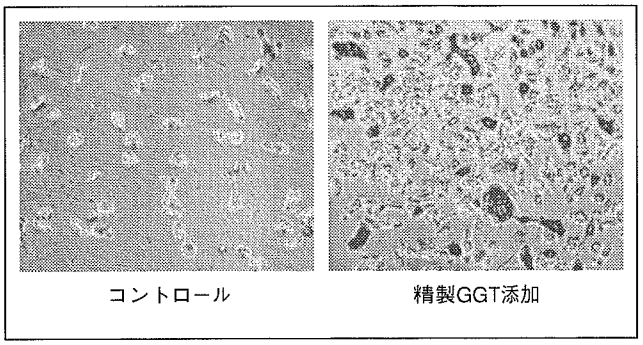

図2．骨髄培養系に精製GGTを添加して誘導された破骨細胞
色の濃い大型の細胞が破骨細胞。

ヒトGGTを遺伝子組換え技術で精製し，再度実験してみたが，結果は同じであった。

次に，GGTを加えた骨髄培養に抗GGT抗体(阪大・谷口教授提供)を入れ，その影響について調べてみた。当然といえば当然であるが，破骨細胞形成と骨吸収活性は抑制された。ところが，この抗体は酵素活性を阻害しない。すなわち，酵素活性があるのに破骨細胞形成活性は消失した。このことはGGTの破骨細胞形成能は，われわれが知るところのGGTの酵素活性とは無関係であることを意味している(**図3**)[8]。そこで，アシビシンという酵素活性阻害物質をGGTに修飾させた不活性型のGGTを精製し，それを骨髄培養に加えてみた。**図4**に示したように，不活性型GGTもまた濃度依存的に破骨細胞形成を誘導することが示された。GGTには酵素活性とは別に，破骨細胞形成能という独立した機能をもつことが明らかになった[8]。

GGTは骨髄培養においてRANKLの発現を誘導する

骨吸収因子の多くは最終分化因子であるRANKLの発現誘導因子である。RANKLは骨芽細胞から分泌される膜結合型のタンパクであるが，骨芽細胞の発現する各骨吸収因子の受容体を介してその発現が誘導される。そこで，GGTがRANKLを誘導しているのかどうか調べてみた。RANKLの囮受容体であるオステオプロテジェリン(OPG)という破骨細胞分化抑制タンパクがある[9]。これは生体内でも働いているもので，破骨細胞の分化を負に調節している。このタンパクの欠損マウスはたちまち骨粗鬆症になる[10]。負の調節が働かなくなったので骨吸収が暴走した結果である。このタンパクをGGTで誘導される破骨細胞の系に入れると濃度依存的に細胞数は減少した。すなわち，GGTによる破骨細胞形成はRANKLに依存していると考えられる。実際，GGTで刺激された骨髄培養系ではRANKLのmRNAの発現上昇がみられる(**図5**)。加えて，タンパクレベルの上昇も確認され[8]，GGTはRANKL発現を誘導するタイプの骨吸収因子であ

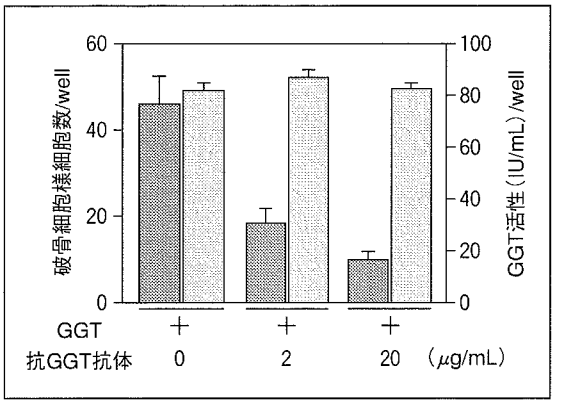

図3．GGTの破骨細胞形成能と酵素活性
GGTによる破骨細胞形成は抗GGT抗体によって用量依存的に抑制されたが，酵素活性は中和されていない。
(文献8より引用・改変)

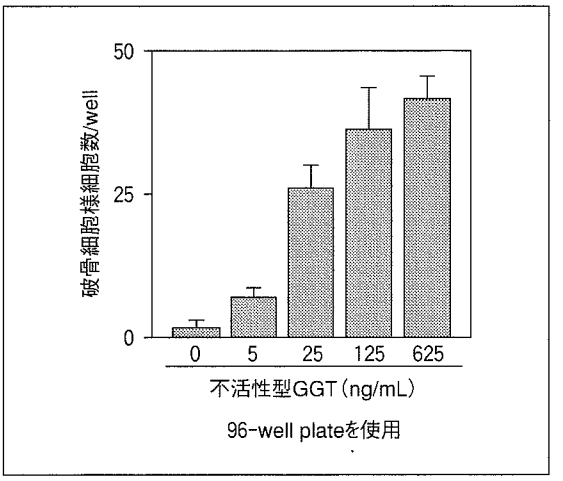

図4．不活性型GGTによる破骨細胞形成
GGTの酵素活性を化学的に不活性化したGGTを作製して骨髄培養系に添加しても，破骨細胞の形成が起こる。
(文献8より引用・改変)

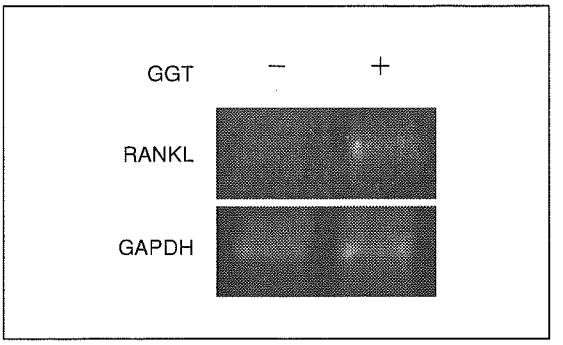

図5．GGTを添加した骨髄培養における破骨細胞分化因子RANKLの発現亢進
(文献8より引用・改変)

ることが示された。

繰り返しになるが，GGTの破骨細胞誘導には酵素活性は関係ない。それならどのようにしてRANKL発現を誘導するのか。それぞれの骨吸収因子は骨芽細胞の膜表面にある特定の受容体に結合して細胞内にRANKL発現のためのシグナルを送る。それならGGTにも受容体があるのではないか。GGTが骨吸収因子かもしれないというのだから，その受容体があるくらい不思議なことではない。

▶ GGTは病的な骨吸収因子 ◀

GGTは実際にはどういうところで骨吸収因子として働いているのか。GGTは先にも述べたように腎臓や肝臓で発現している。そのほか，膵臓や腸上皮，唾液腺などの管腔上皮で発現しているが，およそ骨とは縁がなさそうである。しかし，骨にできたある種の癌だけでなく，関節炎などの炎症でもGGT発現が亢進することがわかってきた。これらについては現在調べている最中で，詳細について記載することができず残念であるが，今のところ病的な環境下で発現する局所的骨破壊因子ではないか，と考えている。

それなら，血中GGT値が高いと骨粗鬆症になるのか。そういう疑問が生じる。骨粗鬆症患者はGGTが高い，という話は聞いたことがない。私たちの研究部ではGGTを過剰発現するトランスジェニックマウスを作製し，その解析を進めている。このマウスの血中GGTは正常マウスの何倍もある。骨量も正常マウスより低下していることがわかった。GGTの過剰発現は確かに骨代謝に影響するようだ。マウスの話だが，GGTは欠損しても過剰になっても骨粗鬆症の要因になるらしい。いずれにしてもその詳細は今後の研究を待つしかない。ヒトの例を探してみると，アルコール依存症の人は高い血中GGT濃度を維持している。こういう人たちの骨量は減少傾向を示すことが知られている[11]。これはアルコールで骨形成の方が抑制されるからだといわれているが，今回のことからGGTが骨吸収側に働いている可能性もあるのではないか。脂肪肝の人でも血中GGTが高いことがある。しかし，骨量が減っているかどうは知らない。誰か調べられたら是非結果を知らせてほしい。

▶ おわりに ◀

古い検査値の解説書には，「GGTはALPと異なり骨疾患で上昇しない」と注釈しているものもある。これはALPが成長期の骨で強く発現しているので検査値を読むときに注意が必要であることに対比させた，いわば「配慮」であったと思われる。その配慮も近頃ではみることがない。おそらく配慮が不要なくらい常識化したからであろう。しかし，今回の研究はその常識に異論を唱える結果となった。ひょっとすると，今度は「常識」を見直すための「配慮」が必要になるかもしれない。

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VEGF receptor 1 signaling is essential for osteoclast development and bone marrow formation in colony-stimulating factor 1-deficient mice

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VEGF receptor 1 (VEGFR-1/Flt-1) is a high-affinity tyrosine kinase (TK) receptor for VEGF and regulates angiogenesis as well as monocyte/macrophage functions. We previously showed that the osteoclast deficiency in osteopetrotic *Csf1*^{op}/*Csf1*^{op} (*op/op*) mice is gradually restored in an endogenous, VEGF-dependent manner. However, the molecular basis of the recovery is still not clear. To examine which VEGFR is important and to clarify how colony-stimulating factor 1 (CSF-1) and VEGF signals interact in osteoclastogenesis, we introduced a VEGFR-1 signaling deficiency (*Flt1*$^{TK-/-}$) into *op/op* mice. The original *Flt1*$^{TK-/-}$ mice showed mild osteoclast reduction without bone marrow suppression. The double mutant (*op/opFlt1*$^{TK-/-}$) mice, however, exhibited very severe osteoclast deficiency and did not have numbers of osteoclasts sufficient to form the bone marrow cavity. The narrow bone marrow cavity in the *op/opFlt1*$^{TK-/-}$ mice was gradually replaced with fibrous tissue, resulting in severe marrow hypoplasia and extramedullary hematopoiesis. In addition to osteoclasts, osteoblasts also decreased in number in the *op/opFlt1*$^{TK-/-}$ mice. These results strongly suggest that the interaction of signals by means of VEGFR-1 and the CSF-1 receptor plays a predominant role not only in osteoclastogenesis but also in the maintenance of bone marrow functions.

osteoblast | hematopoiesis | hematopoietic niche | osteopetrosis | *op/op* mouse

Osteoclasts are terminally differentiated cells derived from the monocyte/macrophage lineage and serve critical functions in bone resorption. The differentiation, activation, and survival of osteoclasts are primarily regulated by colony-stimulating factor 1 (CSF-1)/macrophage colony-stimulating factor, whose biological effects are mediated through a cell surface receptor, CSF-1R/c-Fms (1, 2). The role of CSF-1 in osteoclast biology was first revealed in the osteopetrotic (*Csf1*^{op}/*Csf1*^{op}, hereafter abbreviated as *op/op*) mouse, which has a recessive mutation in the *Csf1* gene (3). The *op/op* mouse exhibits a severe deficiency of osteoclasts, monocytes, and tissue macrophages owing to a lack of CSF-1 function (4). Interestingly, however, the defect is evident only in juvenile mice. Osteoclasts gradually appear in *op/op* bone and correct the osteopetrosis spontaneously. In addition, a single administration of CSF-1 protein resulted in long-term, active bone resorption in *op/op* mice (5, 6). These findings suggest that some alternative factor (or factors) support and maintain osteoclastogenesis in the absence of CSF-1. We demonstrated that the administration of VEGF-A ameliorated osteoclastogenesis and bone resorption and that treatment with an antagonist for VEGF-A suppressed the spontaneous recruitment of osteoclasts in *op/op* mice (7). These results indicate that VEGF is a candidate cytokine to substitute for CSF-1 in the osteoclast development in *op/op* mice.

VEGF-A is a key regulator of physiological angiogenesis and hematopoiesis (8, 9) and has been implicated in the establishment of epiphyseal vascularization and endochondral bone formation (10, 11). VEGF-A belongs to a gene family of growth factors (the VEGF family) that includes VEGF-A, placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (12). Also, an orf virus-derived VEGF, VEGF-E, has been identified (13). VEGF-A has multiple spliced isoforms, including VEGF-A_{120}, VEGF-A_{164}, and VEGF-A_{188}, in mice (12). VEGF-A binds to tyrosine kinase (TK) receptors, VEGF receptor 1 (VEGFR-1/Flt-1) and VEGFR-2 (Flk-1/KDR), subsequently serving as key mediators for angiogenesis (14, 15). PlGF and VEGF-B bind only to VEGFR-1. VEGF-C and VEGF-D bind to VEGFR-3 and regulate lymphatic angiogenesis. VEGF-E is a specific ligand to VEGFR-2 (13–15).

VEGFR-1 is expressed in monocytes and regulates their activation and chemotaxis (16, 17). We also revealed that monocyte/macrophage lineage cells including osteoclasts express VEGFR-1 (7, 18), indicating that, at the very least, VEGFR-1 is involved in osteoclastogenesis. In addition, recent studies suggested that VEGFR-2 is also expressed to some extent in mature osteoclasts (19, 20). To determine the function of the VEGF–VEGFR system in osteoclast development and activity, we introduced a VEGFR-1 TK domain-deficient mutation (*Flt1*$^{TK-/-}$) (21) into *op/op* mice. The double mutant *op/opFlt1*$^{TK-/-}$ mice showed an extensive osteoclast deficiency compared with *op/op* mice and could not recruit numbers of osteoclasts sufficient to expand the marrow cavity, resulting in bone marrow fibrosis and extramedullary hematopoiesis.

Materials and Methods

Mice. The *Flt1*$^{TK-/-}$ mice used in this study are described in ref. 21. Female *Flt1*$^{TK-/-}$ mice with a C57BL/6 background were mated with male *op/op* homozygous mice (The Jackson Laboratory) having the B6C3Fe-*a/a-Csf1*^{op}/*Csf1*^{op} background. Double heterozygotes (*op/+Flt1*$^{TK+/-}$) of the subsequent generation were used for further breeding. The resulting mice, which were

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Abbreviations: CSF-1, colony-stimulating factor 1; VEGFR, VEGF receptor; PlGF, placenta growth factor; TK, tyrosine kinase; TRAP, tartrate-resistant acid phosphatase; ALP, alkaline phosphatase; rh, recombinant human.

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deficient in one gene and heterozygote for another, were used for breeding in parallel. Mice with the *op/op* phenotype were identified by the absence of incisor eruption and/or PCR analysis of tail DNA samples. Mice with the $Flt1^{TK-/-}$ genotype were identified by PCR analysis and/or Southern blot analysis of the same DNA samples as described in ref. 21. The offspring were *op/op* and double mutant $op/opFlt1^{TK-/-}$ and served as the subjects in this study. All animal experiments were approved by the National Center for Geriatrics and Gerontology's institutional animal experimentation committee.

Histological Analysis. Mice (4–24 wk old) were anesthetized and perfused with a periodate-lysine-paraformaldehyde (PLP) solution (4% paraformaldehyde/0.01 M $NaIO_4$/0.075 M lysine in 0.05 M phosphate buffer, pH 7.4). Bone, spleen, liver, and kidney organ blocks were postfixed for 10 h in the PLP solution. After being rinsed with the buffer, soft tissues were embedded in paraffin. Bones were decalcified in a 10% EDTA solution in 1 mM PBS (pH 7.4) for 2 wk at 4°C and embedded in paraffin. These samples were sectioned (3- to 7-μm thick) and stained with hematoxylin/eosin or toluidine blue for histological and pathological observations. Longitudinal serial sections of the median portion of whole femora were stained for tartrate-resistant acid phosphatase (TRAP) activity and counterstained with hematoxylin as described in ref. 7. TRAP-positive cells on bone surfaces that contained more than two nuclei were counted as osteoclasts. To identify the type of fibers in the myelofibrosis, sections of $op/opFlt1^{TK-/-}$ femora were stained with Azan stain, silver stain, periodic acid-methenamin-silver stain, Masson's trichrom stain, van Gieson stain, and phophotungstic acid-hematoxylin stain to diagnose the changes in $op/opFlt1^{TK-/-}$ bone marrow.

Immunohistochemistry. Sections of several tissues of 4- and 8-wk-old mice were immunohistochemically stained with rat anti-mouse F4/80 Ab for detection of mature macrophages (22) and anti-mouse alkaline phosphatase (ALP) Ab (23). The primary Abs were detected with the streptavidin–biotin complex by using a Vectastain kit (Vector Laboratories) for macrophages and osteoblasts according to the manufacturer's instructions. Normal rabbit IgG was used as a control for the antibodies.

Cytokine Injections. Five micrograms of recombinant human (rh) CSF-1 (provided by Morinaga Milk Industry, Tokyo), recombinant mouse $VEGF_{120}$, rhPlGF (both from R & D Systems), or orf virus-derived VEGF-E (13) was i.p. injected into 21-d-old *op/op* and $op/opFlt1^{TK-/-}$ mice. The mice were killed 4 d after the injections. As a control, vehicle (0.1 M PBS) was injected similarly as above.

A group of 7-wk-old *op/op* mice received three consecutive injections of 5 μg of VEGFR-1/Fc chimeric protein (R & D Systems) under the conditions described above. Finally, three consecutive injections of 5 μg of rhCSF-1 were given to 7-wk-old $op/opFlt1^{TK-/-}$ mice at 24-h intervals. All of these mice were killed 5 d after the last injection.

Osteoclast Formation *in Vitro*. Spleen cells of 4-wk-old mice were passed through a Sephadex G-10 column (Amersham Pharmacia Biotech) as described in ref. 7. Cells were plated in 96-well plates at a density of 10^5 cells per well onto confluent OP9 stromal cells, which were established from *op/op* mouse calvaria (24), and cultured for 6 d in α-MEM supplemented with 10% FBS in the presence of 10 ng/ml rhCSF-1, 50 ng/ml recombinant mouse $VEGF_{120}$, VEGF-E, and rhPlGF. The cultures were fixed with 4% paraformaldehyde and stained for TRAP. TRAP-positive multinucleated (three or more nuclei) cells were scored as osteoclasts under the microscope.

Statistical Analysis. Values are expressed as the mean ± standard deviation. Significant differences between groups were determined with Student's *t* test in STAT VIEW 5.0 (SAS Institute, Cary, NC).

Results and Discussion

A Mild Reduction of Osteoclasts in $Flt1^{TK-/-}$ Mice. We previously showed that endogenous VEGF-A substituted for CSF-1 in osteoclast development during the adult stage in CSF-1-deficient *op/op* mice and that osteoclasts expressed VEGFR-1 (7). Furthermore, ovariectomized *op/op* mice exhibited an increased number of osteoclasts accompanied by up-regulation of VEGF-A and VEGFR-1 mRNA expression (25). Thus, before crossing the *op/op* mice with $Flt1^{TK-/-}$ mice, which undergo a basically normal development including angiogenesis (21), we examined the effects of VEGFR-1 signaling deficiency on the osteoclast formation *in vivo* by using $Flt1^{TK-/-}$ mice. These mice displayed a mild reduction in numbers of TRAP-positive multinucleated osteoclasts and bone trabeculae just below the growth plate in long bones compared with that in WT mice (Fig. 1). Although the number of osteoclasts was sufficient for bone morphogenesis, these results suggest that VEGFR-1 signaling is partly implicated in physiological osteoclastogenesis.

***op/op* Mice Lacking a VEGFR-1 TK Domain Show Severe Bone Marrow Cavity Occlusion.** To clarify the roles of VEGFR-1 in osteoclast formation in more detail, the $Flt1^{TK-/-}$ mice were bred with the *op/op* mice (Fig. 2*A*). The *op/op* mice lacking the VEGFR-1 TK domain ($op/opFlt1^{TK-/-}$) showed no significant difference in body weight or skeletal size compared with *op/op* mice (data not shown). F4/80-positive macrophage numbers were similarly reduced in marrow, liver, spleen, and kidney in both *op/op* and $op/opFlt1^{TK-/-}$ mice (Fig. 2*B*). However, the limb bones in 2-wk-old $op/opFlt1^{TK-/-}$ mice exhibited a more severe osteopetrosis with a decrease in the number and size of osteoclasts compared with those in *op/op* mice (Fig. 2*C*).

Next, we examined histological changes of femora in mice aged 4–24 wk. In *op/op* mice, the original osteopetrosis gradually ameliorated and marrow cellularity increased between the ages of 8 and 24 wk (Fig. 3*A*), as shown previously (7). In contrast, in $op/opFlt1^{TK-/-}$ mice, the osteoclastic bone resorption did not recover throughout the observation period (6 mo). The osteopetrotic phenotype remained in $op/opFlt1^{TK-/-}$ mice even at the 24-wk-old stage, although the thickened growth plate that is one of the features of osteopetrotic mice had been replaced with bone trabeculae (Fig. 3*A*). These results suggest that the VEGF-dependent osteoclastic bone resorption system does not function sufficiently in $op/opFlt1^{TK-/-}$ mice.

Then, we compared the number of osteoclasts in femora of *op/op* and $op/opFlt1^{TK-/-}$ mice. Small numbers of TRAP-positive osteoclasts were observed in 4-wk-old *op/op* femora, and numbers gradually increased during the observation period (Fig. 3*B*). Although osteoclasts were hardly detectable in 4-wk-old $op/opFlt1^{TK-/-}$ femora, a small number appeared in 8-wk-old bone. The number of osteoclasts transiently increased to a lesser extent at 16 wk, returning to a barely detectable level again at 24 wk (Fig. 3*B*). In age-matched WT mice, 100 or 200 osteoclasts were observed during the observation period. These results clearly indicate that the TK domain in VEGFR-1 plays a pivotal role in the recruitment of osteoclasts in mice with a CSF-1-deficient background. The transient appearance of osteoclasts in the $op/opFlt1^{TK-/-}$ bones, however, might suggest other signaling pathway(s).

Exogenous VEGFs Rescue Osteopetrosis in $op/opFlt1^{TK-/-}$ Mice. Given our previous observation that the administration of neutralized Ab against VEGF-A completely inhibited osteoclast development in *op/op* mice (7), VEGFR-2 may be responsible

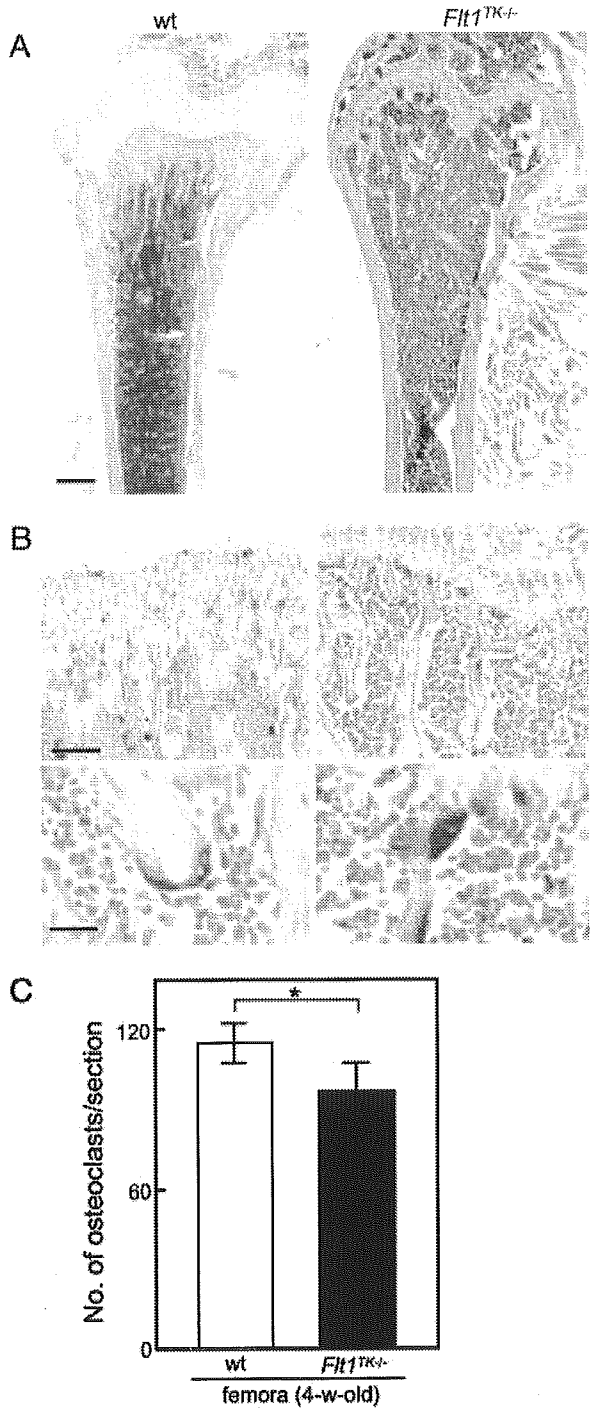

Fig. 1. Bone histology and number of osteoclasts in femora of 4-wk-old WT and $Flt1^{TK-/-}$ mice. (*A*) Longitudinal sections of femora were stained with hematoxylin/eosin. (Scale bar: 0.5 mm.) (*B*) Red-stained cells on the bone trabeculae are TRAP-positive osteoclasts. (*Lower*) High-magnification image of multinucleated osteoclasts. (Scale bars: *Upper*, 100 μm; *Lower*, 25 μm.) (*C*) The numbers of osteoclasts in the sections of the median portion of femora were counted. The TK domain deficiency showed a mild reduction in number of osteoclasts (*, $P < 0.05$).

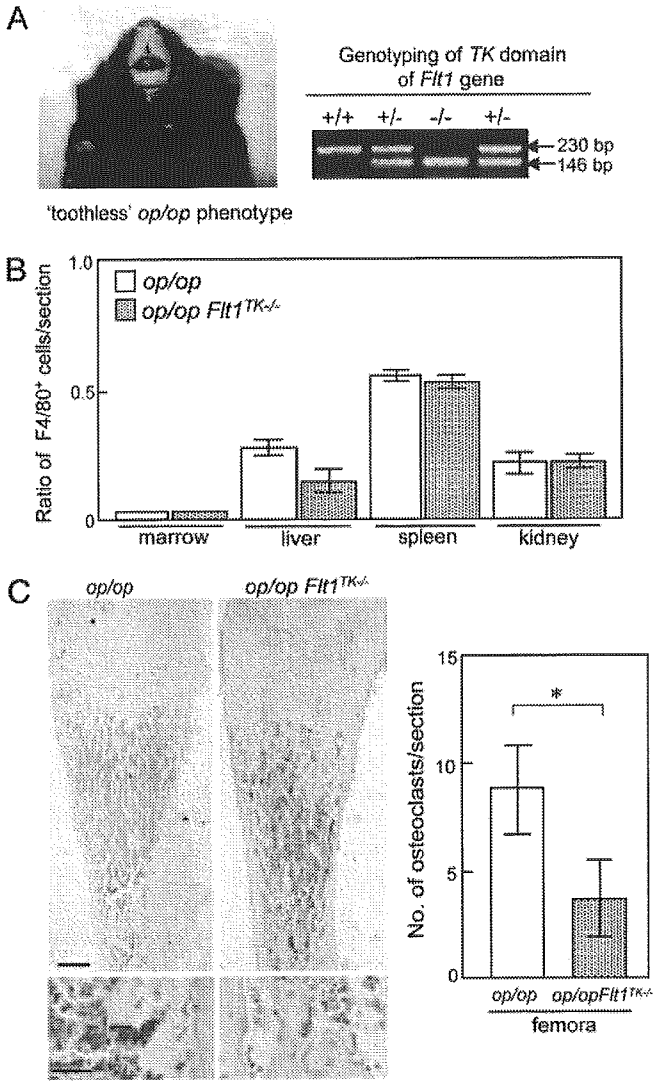

Fig. 2. Phenotype of $op/opFlt1^{TK-/-}$ mice. (*A*) Double mutant $op/opFlt1^{TK-/-}$ mice exhibit an *op/op* toothless phenotype with the genotype of $Flt1^{TK-/-}$ (−/−) from a litter resulting from the interbreeding of $op/+Flt1^{TK+/-}$ and $op/+Flt1^{TK+/-}$ mice. Tail DNA was isolated and analyzed by PCR according to our previous study (21). (*B*) The ratio of numbers of macrophages with F4/80 immunoreaction in various tissues of $op/opFlt1^{TK-/-}$ mice to WT mice. (*C*) The $op/opFlt1^{TK-/-}$ femur exhibits a more severe osteopetrosis. The small red spots are osteoclasts. TRAP-positive cells containing nuclei, as shown in the high-magnification image, were counted as osteoclasts. The number of osteoclasts in the sections of 2-wk-old $op/opFlt1^{TK-/-}$ mice femora significantly (*, $P < 0.01$) decreased compared with that in age-matched *op/op* mice. (Scale bars: *Upper*, 0.5 mm; *Lower*, 25 μm.)

for the mild and transient recruitment of osteoclasts in $op/opFlt1^{TK-/-}$ mice. To test this hypothesis, we used ligands specific for VEGFR-1 and VEGFR-2 in *op/op* and $op/opFlt1^{TK-/-}$ mice. rhCSF-1 was used as a control. The administration of rhPlGF, a VEGFR-1-specific ligand (26, 27), efficiently restored osteoclast formation in *op/op* mice but not in $op/opFlt1^{TK-/-}$ mice (Fig. 4*A*), although the osteoclasts were mostly small, and the degree was half that with rhCSF-1 (Fig. 4*A*). These results support our basic idea that VEGFR-1 is an important mediator for osteoclast formation. We next examined whether VEGF-E, a VEGFR-2-specific ligand, leads to osteoclastogenesis in $op/opFlt1^{TK-/-}$ mice. The VEGF-E administrations induced small osteoclasts in both *op/op* and $op/opFlt1^{TK-/-}$ mice. Similar results were obtained from the administration of $VEGF_{120}$, which binds both receptors (Fig. 4*A*).

These activities of VEGFs were also observed in an *in vitro* osteoclast formation assay in which spleen cells were cocultured with OP9 osteoclastogenesis-supportive stromal cells (Fig. 4*B*).

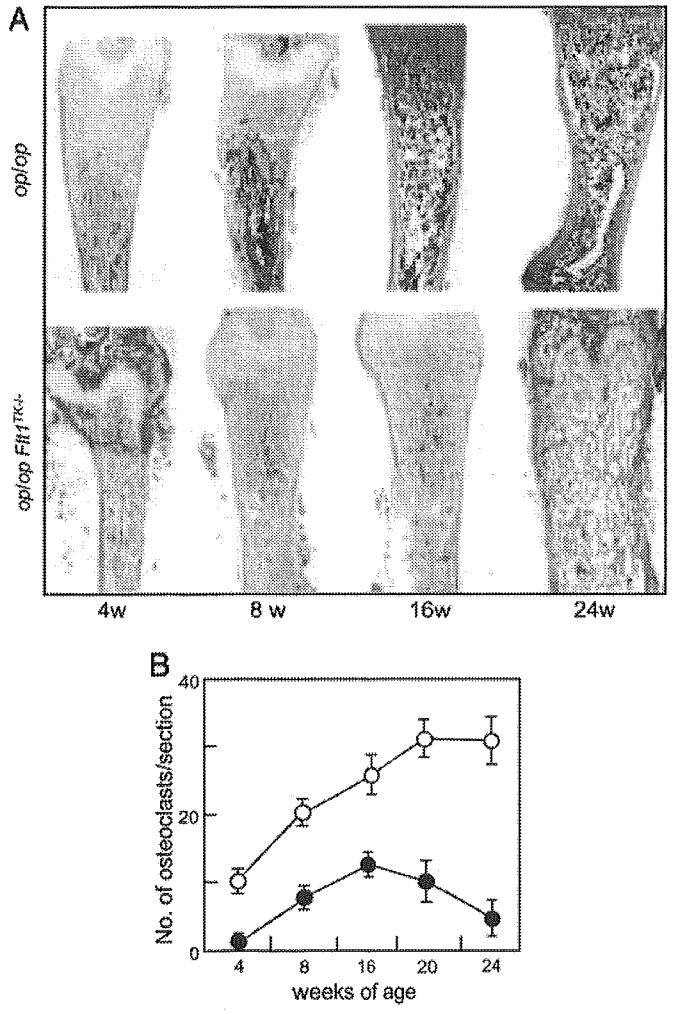

Fig. 3. *op/opFlt1*$^{TK-/-}$ mice cannot recruit enough osteoclasts to form bone marrow. (*A*) Growth changes of femora in *op/op* and *op/opFlt1*$^{TK-/-}$ mice. Longitudinal sections of femora were stained with hematoxylin/eosin. Bone trabeculae in *op/op* mice decreased with aging, whereas those of *op/opFlt1*$^{TK-/-}$ mice remained until 24 wk of age. (*B*) Changes in numbers of TRAP$^+$ osteoclasts in *op/op* (open circles) and *op/opFlt1*$^{TK-/-}$ (filled circles) mice.

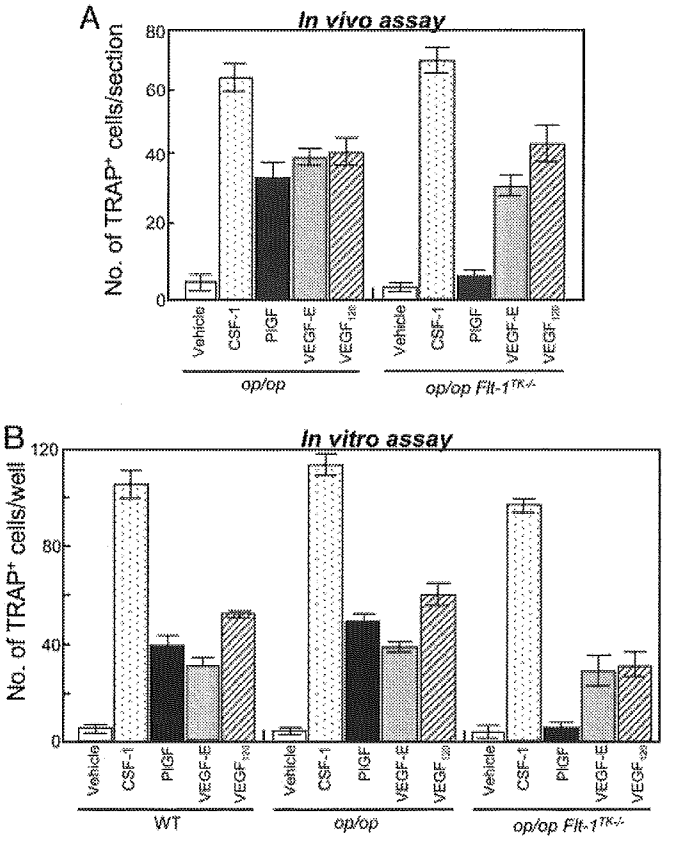

Fig. 4. Osteoclast-induction activity of rhCSF-1, recombinant mouse VEGF$_{120}$, rhPlGF, and VEGF-E in both mutant mice. (*A*) rhCSF-1 and VEGFs were injected in a single dosage of 5 μg into <3-wk-old *op/op* and *op/opFlt1*$^{TK-/-}$ mice, and mice were killed 4 d after the injection. Osteoclasts in longitudinal sections of the median portion of femora were counted. (*B*) *In vitro* assay of osteoclastogenic activity of rhCSF-1 and various VEGFs. Spleen cells of *op/op* and *op/opFlt1*$^{TK-/-}$ mice were cultured for 6 d with OP9 stromal cells in the presence of each cytokine in 96-well plates. The cultures were stained for TRAP activity, and TRAP-positive cells were counted.

Although PlGF-induced osteoclast-like cells were smaller than those induced by CSF-1 treatment, they have multiple nuclei. The cells induced with VEGF-E were the smallest, mostly with only a few nuclei. These features were essentially the same without OP9 feeder cells (see the supporting information, which is published on the PNAS web site); however, the survival rate of the cells was lower compared with that in the OP9 feeder system. These findings suggest that VEGFR-2 might play some role in the development of osteoclasts in *op/op* background mice. In our previous study, however, VEGFR-2 was under detectable levels in monocyte/macrophage lineage cells (18). A low level of VEGFR-2 might induce a differentiation signal, or VEGFR-2 could be up-regulated during the culture period, because a recent study showed that VEGFR-2-expression in monocytes/macrophages, initially undetectable, was induced by VEGF stimulation (28).

Our findings indicate that exogenous PlGF has an osteoclastogenic activity, raising the question of whether endogenous PlGF (or VEGF-B) plays a role in the activation of VEGFR-1 and up-regulates osteoclastogenesis *in vivo*. However, recent studies provided evidence that PlGF-deficient mice are normally developed and healthy without clear abnormality (29). VEGF-B-deficient mice are also basically healthy, with normal morphology except in the heart. Some VEGF-B$^{-/-}$ mice showed an enlarged heart (30, 31). These findings suggest that endogenous PlGF and VEGF-B have only a minor effect, if any, on the osteoclastogenesis by means of activation of VEGFR-1 and that the major signal is generated by means of endogenous VEGF-A.

Another question is why the endogenous VEGF-A cannot rescue osteoclastogenesis by means of VEGFR-2 in *op/opFlt1*$^{TK-/-}$ mice. We suggest two reasons. The first is a quantitative point. Concentration of endogenous VEGF-A is known to be significantly low compared with that of exogenous VEGF-A. Furthermore, the affinity of VEGFR-2 to VEGF-A is one order weaker than that of VEGFR-1 (14, 27). Thus, the endogenous VEGF-A may not be sufficient to stimulate VEGFR-2 at high levels. The second is a qualitative point. Our *in vitro* studies (Fig. 4*B*) indicate that the VEGFR-2-specific ligand induced osteoclast-like cells with only a few nuclei. Therefore, VEGFR-2 signaling might be qualitatively insufficient to generate a full differentiation signal in osteoclast precursor cells.

Myelofibrosis in *op/opFlt1*$^{TK-/-}$ Mice. Another striking feature of *op/opFlt1*$^{TK-/-}$ mice is the significant increase in fibrous tissues instead of hematopoietic cells in the marrow cavity. In 8-wk-old

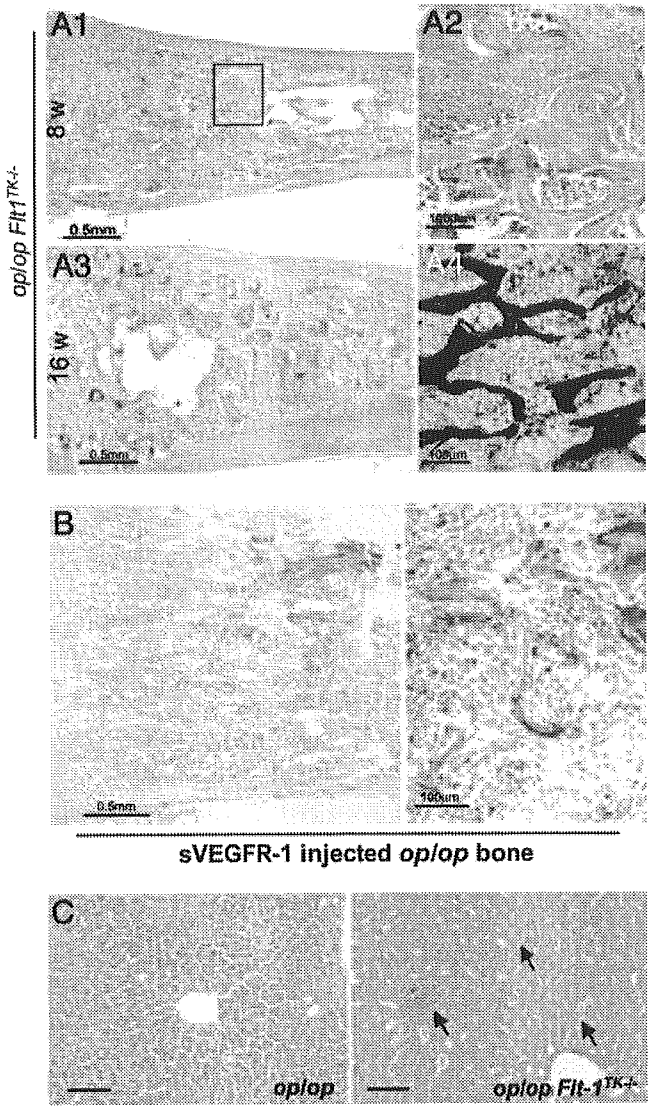

Fig. 5. Severe myelofibrosis in *op/opFlt1*$^{TK-/-}$ mice. (*A1* and *A2*) Myelofibrosis was found in the diaphysial region of the marrow cavity in 8-wk-old mice. A high-magnification image of the boxed area shows the junction of normal marrow and myelofibrosis. (*A3*) Myelofibrosis filled the bone marrow space in 16-wk-old mice. (*A4*) A high-magnification image shows silver staining for myelofibrosis. (*B*) TRAP and histology of the femur of 8-wk-old *op/op* mice treated with soluble VEGFR-1/Fc chimeric protein, indicating very few osteoclasts. (*C*) Hematoxylin/eosin-stained livers of *op/op* and *op/opFlt1*$^{TK-/-}$ mice. Small hematopoietic foci were observed in *op/opFlt1*$^{TK-/-}$ mice (*Right*, arrows) but not in *op/op* mice (*Left*). (Scale bars: 100 μm.)

mice, fibrous tissue was initially observed in the diaphysial regions of the femora, although hematopoietic cells still occupied the intratrabecular spaces in the epiphysial region (Fig. 5*A1* and *A2*). With aging, the fibrous tissue gradually expanded to the whole marrow cavity, resulting in marked decreases in marrow cellularity, including osteoclasts (Fig. 5*A3*). Histological analysis indicated that the fibrous tissue consisted of reticular fiber-like fibrils (Fig. 5*A4*). Furthermore, we found that the phenotype obtained on administration of soluble VEGFR-1 chimeric protein, an efficient VEGF inhibitor, into *op/op* mice mimicked the marrow phenotype of *op/opFlt1*$^{TK-/-}$ mice (Fig. 5*B*).

Recent studies suggest that a portion of hematopoietic stem cells (HSCs) express VEGFR-1 (32) and that HSCs require VEGFR-1 signaling for their recruitment and mobilization in marrow (33). According to these reports, the decreased marrow

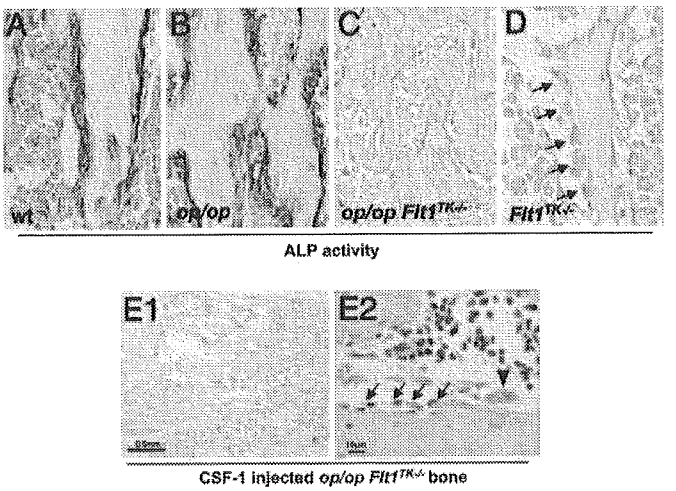

Fig. 6. Immunohistochemistry of ALP activity in four mouse genotypes: WT, *op/op*, *op/opFlt1*$^{TK-/-}$, and *Flt1*$^{TK-/-}$. (*A–D*) Cells stained brown are ALP-positive osteoblasts. The ALP activity of the *op/opFlt1*$^{TK-/-}$ and *Flt1*$^{TK-/-}$ mice is extremely weak compared with that of the WT and *op/op* mice. Although the ALP activity is weak, the osteoblasts in the *Flt1*$^{TK-/-}$ mice retain the morphology of active-phase cells (*D*). However, in the *op/opFlt1*$^{TK-/-}$ mice, most osteoblasts disappeared from the bone trabeculare. (*E*) TRAP activity (*E1*) and histology (*E2*) of the bone marrow in the femur of 8-wk-old *op/op* mice treated with rhCSF-1. Administration of rhCSF-1 prevented changes in the marrow and osteoblast reduction in the *op/opFlt1*$^{TK-/-}$ mice. Arrows, osteoblasts; arrowhead, osteoclast.

cellularity in *op/opFlt1*$^{TK-/-}$ mice might be due to defective HSCs. However, the single-gene mutant *Flt1*$^{TK-/-}$ mice showed no apparent defect in marrow cellularity (Fig. 1*A*). Furthermore, a number of small hematopoietic foci were found in the liver of *op/opFlt1*$^{TK-/-}$ mice (Fig. 5*C*). Taken together, these findings indicate that HSCs do exist even in the *op/opFlt1*$^{TK-/-}$ mice and are functional for extramedullary hematopoiesis.

A Possible Intercommunication Between Osteoclasts and Osteoblasts. Myelopoiesis is supported by marrow stromal cells, including osteoblasts, which produce various osteogenic and hematopoietic growth factors (34). Osteoblast-deficient mice, owing to a lack of the *Runx2/Cbfa1* gene, which encodes a transcription factor for osteoblastogenesis, exhibit an absence of marrow cells (35). Osteoblast deficiency induced by different genetic approaches also arrests marrow hematopoiesis and establishes extramedually hematopoiesis (36). Increases in the number of osteoblasts correlate with the establishment of hematopoietic niches (37, 38). Therefore, next we examined the activity of osteoblasts in the *op/opFlt1*$^{TK-/-}$ mice.

Immunostaining for ALP in the bone sections of *op/opFlt1*$^{TK-/-}$ mice revealed a remarkable reduction in the immunoreaction compared with that of *op/op* and WT mice (Fig. 6 *A–C*). Osteoblasts were significantly decreased on the surface of bone trabeculae, adjacent to myelofibrosis (Fig. 6*C*). The down-regulation of osteoblast activity may disrupt the hematopoiesis-supportive microenvironment in *op/opFlt1*$^{TK-/-}$ mice, resulting in a reduction in marrow cellularity and an increase in fibrosis. Because marrow hematopoiesis was weakly initiated in young *op/opFlt1*$^{TK-/-}$ mice, we tested for ALP activity of osteoblasts in *Flt1*$^{TK-/-}$ mouse bone. Although many osteoblasts were observed in *Flt1*$^{TK-/-}$ mice, ALP activity was extremely weak (Fig. 6*D*). Taken together, our data provide genetic evidence that VEGFR-1 signaling is important for osteoblast activity during bone formation.

Bone undergoes remodeling through the coordinated process of bone resorption and bone formation to maintain bone mass.

It is considered that this harmonious balance is modulated by coupling paracrine signaling between osteoclasts and osteoblasts (for review, see ref. 39). Thus, we hypothesized that the survival of osteoblasts in $Flt1^{TK-/-}$ mice may be supported by the existence of osteoclasts. To test this, we lastly examined whether induction of osteoclasts would rescue the hypoplastic marrow in $op/opFlt1^{TK-/-}$ mice. Administration of rhCSF-1 to 7-wk-old $op/opFlt1^{TK-/-}$ mice restored not only osteoclasts but also osteoblasts (Fig. 6*E*). Moreover, rhCSF-1 treatment also prevented marrow alterations (Fig. 6*E2*). These findings suggest that osteoclasts are implicated in the survival of osteoblasts and that osteoblasts are crucial for construction of the marrow hematopoiesis-supportive microenvironment as described in refs. 36 and 37. Simultaneously, our findings demonstrate that CSF-1 plays an important role not only in bone remodeling but also in the organization of marrow structure.

In conclusion, we provided anatomical and genetic findings to show the importance of the interaction of VEGFR-1 signaling and CSF-1 receptor signaling in mice. Lack of these signals induces a severe alteration in bone and marrow structure. These findings may contribute to further understanding of the interaction between bone cells and marrow cells.

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REVIEW : JAOB : Lion Dental Research Award :

Osteoclast : forming Activity of Vascular Endothelial Growth Factor

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Key words : osteoclast : VEGF : VEGF receptor : Flt : 1 : Flk : 1

Abstract : Colony : stimulating factor : 1 : CSF : 1 : is an essential regulator of the differentiation, proliferation and survival of macrophage lineage cells including bone : resorbing osteoclasts. We have demonstrated that vascular endothelial growth factor : VEGF :, a known angiogenic factor, can act as a substitute for CSF : 1 function in osteoclastogenesis through the VEGF receptor : 1. Osteopetrotic $Csf1^{op}$: $Csf1^{op}$: op : op : mice exhibit severe osteoclast deficiency owing to the lack of CSF : 1 function. However, the deficiency is gradually reversed with aging, suggesting the existence of an alternative factor supporting osteoclastogenesis. We have found that the administration of VEGF to op : op mice induces a sufficient number of osteoclasts to ameliorate the osteopetrosis. Estrogen deficiency induces the acceleration of osteoclastic bone resorption mediated by the upregulation of bone : resorbing factors including CSF : 1. Ovariectomized op : op mice exhibited upregulation of VEGF expression and an increase in number of osteoclasts. VEGF antagonists inhibited both spontaneous osteoclast recruitment in the aging op : op mice and estrogen deficiency : dependent increases in osteoclasts in OVX : op : op mice. These results clearly demonstrate an ability of osteoclastogenic activity of VEGF.

Introduction

: Osteoclast biology has been extensively studied over the last decade. It has been established that hematopoietic growth factor colony : stimulating factor : 1 : CSF : 1, also known as M : CSF : is essential for the proliferation, differentiation, and survival of osteoclasts derived from monocyte : macrophage lineage cells[1,2]:. The biological effects of CSF : 1 are mediated through a cell : surface tyrosine kinase receptor c : Fms, which is one of the eight members of the platelet : derived growth factor receptor : PDGFR : family[3]:. The critical role of CSF : 1 in osteoclastogenesis has been proven in studies using osteopetrotic $Csf1^{op}$: $Csf1^{op}$: op : op : mice. Mice homozygous for a recessive op mutation on chromosome 3 exhibit a severe deficiency of osteoclasts, monocytes and tissue macrophages owing to a lack of functional CSF : 1[4 : 6]:. Yoshida, et al.[7]: revealed that the loss of CSF : 1 function in op : op mice is caused by a point mutation within the cording region of the Csf1 gene. The administration of the recombinant human CSF : 1 : rhCSF : 1 : reversed the defects in op : op mice[8 : 10]:. The expression of c : Fms in osteoclasts demonstrated the direct action of CSF : 1 on osteoclast lineage cells[11,12]:. However, severe osteopetrosis in op : op mice is evident only in juvenile mice. With aging, cells stained by tartrate : resistant acid phosphatase : TRAP :, an osteoclast marker, appear spontaneously in op : op mice bones and correct the osteopetrosis. In addition, only a single dose of rhCSF : 1 : : 5 : g : body : is sufficient to induce not only osteoclastogenesis but also continued

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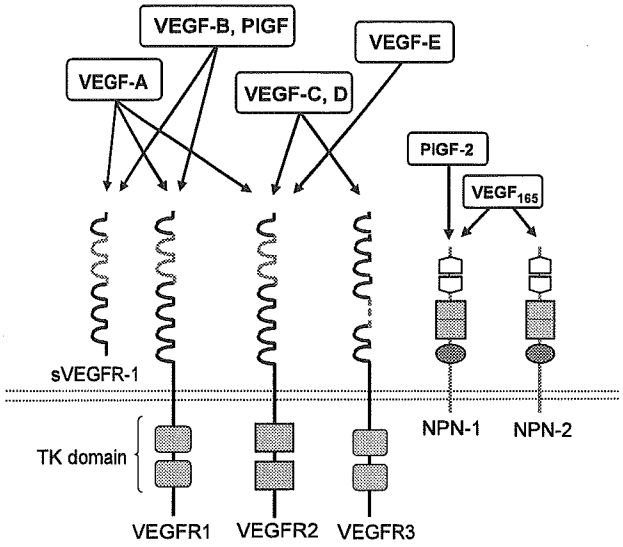

Fig. 1 : VEGF families and their interactions with VEGF receptors. Osteoclasts and preosteoclasts express both VEGFR1 and VEGFR2.

active bone resorption in op/op mice[13,14]. These results suggest the existence of an alternative factor supporting osteoclastogenesis and survival in op/op mice.

Although evidence from op/op mice reveals an essential role for CSF/1 in osteoclast biology, it simultaneously raises the question : what induces osteoclastogenesis in op/op mice?. Granulocyte/macrophage colony/stimulating factor (GM/CSF) has a function similar that of CSF/1 in the development of macrophage linage cells. However, GM/CSF is not responsible for the correction of osteoclast deficiency in the op/op mice[15,16]. We have previously demonstrated that congenital osteoclast deficiency in op/op mice can also be ameliorated by administration of a recombinant human vascular endothelial growth factor (rhVEGF)[17,18].

VEGF and Its Receptors

VEGF is a key regulator of the growth and differentiation of vascular and lymphatic endothelial cells[19], and is also known as vascular permeability factor (VPF)[20]. VEGF belongs to the PDGF supergene family and includes several members including VEGF/A, placenta growth factor (PlGF), VEGF/B, VEGF/C, and VEGF/D. In addition, Orf/virus/derived VEGF/like polypeptide, VEGF/E, has been identified[21]. Human VEGF/A has multiple spliced isoforms including $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$[22-24]. In mice and rats, VEGF/A isoforms are shorter by one amino acid[19,25]. $VEGF_{121}$ fails to bind to heparin, while $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ are heparin/binding proteins[26]. Recently, two other splice variants, $VEGF_{145}$ and $VEGF_{183}$, were identified in humans[27,28].

VEGF receptor 1 (VEGFR1/Flt/1) and VEGF receptor 2 (VEGFR2/Flk/1/KDR) are high/affinity receptos for VEGF/A and function as key mediators for angiogenesis (Fig. 1). These receptors have seven immunoglobulin (Ig)/like domains in their extracellular regions and an 70 amino/acid/long tyrosine kinase (TK) domain in the cytoplasmic regions[29-32]. The fundamental structure of VEGFRs is very similar to that of PDGFR family members such as PDGFR/c/Fms/c/kit/Flt/3, although the PDGFRs have five instead seven Ig/like domains in their extracellular domains. VEGFR1 has a high affinity for $rhVEGF_{165}$[33] and as a decoy receptor for VEGF negatively regulates, at least in some circumstances, angiogenesis[34]. In addition, the VEGFR1 gene encodes an alternatively spliced soluble form of VEGFR1 (sVEGFR1) lacking the seventh Ig/like domain and the cytoplasmic TK domain[29,31,35]. sVEGFR1 also has a high affin-

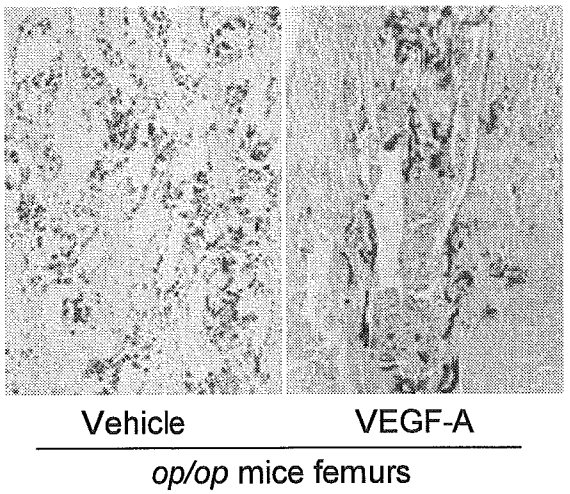

Fig. 2 " Exogenous VEGF'A administration in op'op mice recovering osteoclastogenesis. Cells colored black in the photograph on the right are osteoclasts induced by VEGF'A.

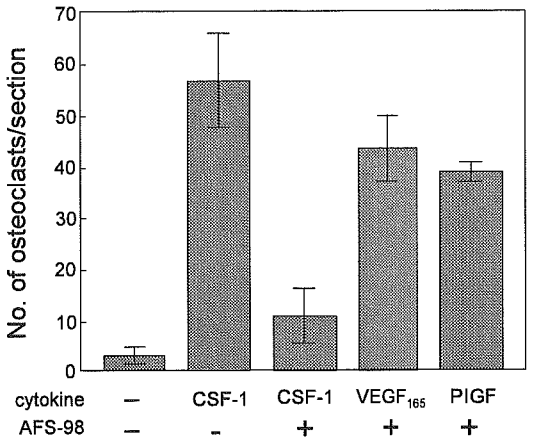

Fig. 3 " AFS98 anti'c'Fms mAb was administered at a dose of 750 "g'body at both 2h before and after 24h after cytokine administration. The numbers of TRAP' cells in the longitudinal sections of whole femurs were counted.

ity for VEGF'A. Although VEGFR2 has a lower affinity for VEGF than does VEGFR1[36]", it serves as a major signaling receptor for endothelial cell proliferation and differentiation. The homozygous deletion of any of these receptor genes in mice results in embryonic lethality attributable to deficiencies or abnormalities in vasculogenesis and angiogenesis[37'39]". In addition to these receptors, $VEGF_{165}$'specific receptor, neuropilin'1 " *NPN*'1 ", has been identified[40]". *NPN*'1 is a known receptor for the semaphorin'collapsin family involved in neuronal cell guidance " it enhances the binding activity of $VEGF_{165}$ to VEGFR2 and $VEGF_{165}$'mediated chemotaxis. Interestingly, it has been reported that VEGFR1 is expressed in monocyte'macrophage lineage cells and is involved in signal transduction for cell migration[41,42]".

VEGF Rescues Osteoclast'deficiency in op'op Mice

" To date, VEGF is the only cytokine, excluding CSF'1, which is able to induce osteoclasts in op'op mice by exogenous administration " Fig. 2 ". This discovery has revealed the existence of a unique type of redundancy in cytokine signaling through different ligand'receptor systems in osteoclastogenesis. Dose'response studies demonstrated that a single injection of 5 "g of $rhVEGF_{165}$ is sufficient to correct osteopetrosis in op'op mice[18]". Either $rhVEGF_{121}$ or rhPlGF'1, a specific ligand of VEGFR1, was effective at inducing osteoclastogenesis[17]". The number of osteoclasts induced by any of these factors was "70 " of that induced by rhCSF'1. *N*eutralization with anti'c'Fms antibody significantly decreased the rhCSF'1'dependent osteoclast induction in op'op mice, but not the rhVEGF'A and rhPlGF'dependent osteoclastogenesis " Fig. 3 ", suggesting the presence of VEGF'A'specific receptors on osteoclast precursors. We have detected the expression of VEGFR1, but not VEGFR2, on the osteoclasts in both normal and op'op mice[17,18]". $VEGF_{121}$ does not bind *NPN*'1[43]". PlGF'1 binds VEGFR1, but not VEGFR2 or *NPN*'1[42'44]". Our results, showing that the osteoclastogenic activities of $rhVEGF_{121}$ and rhPlGF'1 are comparable to that of $rhVEGF_{165}$, support the hypothesis that the response of osteoclast precursors to VEGF is directly mediated, at least in part, by VEGFR1. *N*akagawa, et al.[45]" demonstrated that VEGFR2 is directly involved in osteoclastic bone resorption. Recently, we confirmed immunohistochemically the expression of VEGFR2 on op'op osteoclasts. These results do not exclude the possibility that VEGFR2 is also involved in osteoclastogenesis. Further studies will be

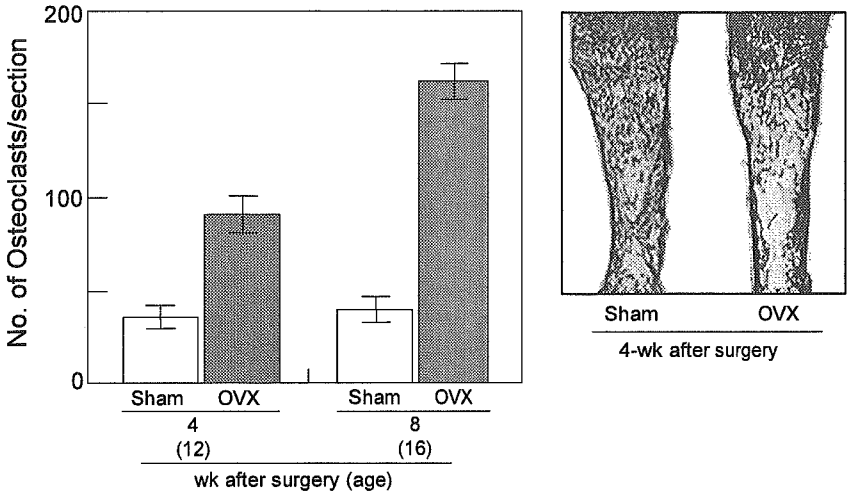

Fig. 4 : OVX-induced osteoclastogenesis and bone resorption in op/op mice. Mice were ovariectomized at 8-week-old, and euthanized at 4 or 8 weeks after surgery.

required to elucidate the VEGFR signaling mechanisms involved in osteoclastogenesis.

: The receptor activator of nuclear factor-B ligand : RANKL : plays a crucial role in the terminal differentiation of osteoclasts in cooperation with CSF-1[46,47]. The osteoclastogenic activity of VEGF was further confirmed by in vitro studies. We cultured mouse bone marrow hematopoietic cells in the presence of rhVEGF$_{165}$ and/or rhRANKL. No TRAP+ cells appeared in the presence of rhVEGF$_{165}$ alone. The combination of rhVEGF$_{165}$ and rhRANKL resulted in the formation of supported TRAP+ cells, although the cell size was smaller than those generated in the presence of rhCSF-1 and rhRANKL[17]. Bone-resorbing activity of these cells has been confirmed by scanning electron microscopy[17].

: Osteopetrosis in op/op mice is progressively corrected with aging owing to spontaneous osteoclast recruitment. We examined whether osteoclast recruitment in op/op mice depends on the endogenous production of VEGF. A significantly larger number of osteoclasts was observed in the femurs of : 2-month-old op/op mice as compared with 2-week-old mice. Five consecutive injections of 100 :g goat anti-VEGF polyclonal antibody at 12-hr intervals significantly decreased the osteoclast number in 2-month-old mice, indicating that VEGF is responsible for spontaneous osteoclast recruitment in the absence of functional CSF-1 in op/op mice.

'Osteoporosis' in the op/op Mice

: Estrogen deficiency leads to bone loss through the action of an increased number of osteoclasts. Many studies in ovariectomized : OVX : animals have implicated increases in various cytokines such as CSF-1, IL-1, IL-6, TNF-, and PGE$_2$[48,49]. These cytokines accelerate bone resorption mainly through the proliferation of presoteoclasts. Estrogen replacement in OVX animals suppresses the expression of these cytokines in osteoclastogenesis-supportive bone marrow stromal cells, monocytes, and lymphocytes[48]. In addition, recent studies indicated that high concentrations of TNF- promote osteoclastogenesis independently of RANKL[50,51].

: Over ten years ago, we discovered that OVX led to a large amount of osteoclastogenesis in op/op mice : Fig. 4 :. Although we had not been able to identify the type of factor(s) : that substitute for CSF-1 in OVX-op/op mice, the results suggested that the factor(s) : were modulated by estrogen deficiency. It has been shown that estrogen and other steroid hormones regulate VEGF production in several tissues. Again, we have attempted to identify the regulator of

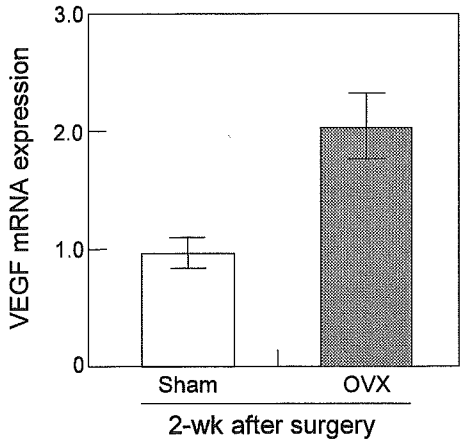

Fig. 5 ' Expression of VEGF mRNA upregulated in OVX'op'op bone. Interestingly, the expression of the mRNAs of VEGFR1 and RANKL was also elevated in OVX'op'op mice.

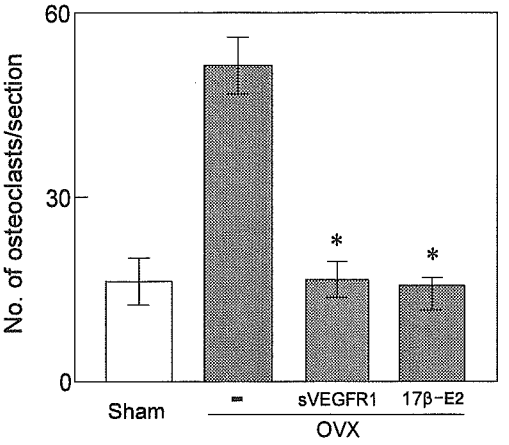

Fig. 6 ' Estrogen deficiency'dependent increase in osteoclast number suppressed by the administration of soluble VEGFR1 chimeric protein or 17 ' 'estradiol ' 17 ' ' E2 '.

osteoclastogenesis in OVX'op'op mice[52]'. VEGF expression was elevated in bone in OVX'op'op mice compared with sham'operated mice ' Fig. 5 '. The enhanced expression of the mRNA for VEGFR1, RANKL and IL'6, but not for TNF' ' and IL'1', was also observed in bones from OVX mice[52]'. It has been shown that IL'6 acts as a stimulator for the production of RANKL and VEGF[53]'. The upregulation of VEGFR1 expression suggests an increase in the number of osteoclast precursors. TNF' ' and IL'1' are mainly produced by monocytes in bone marrow. op'op mice exhibit a severe deficiency of these cells 'therefore, the upregulation of TNF' ' and IL'1' is not observed in op'op mice. Neutralization experiments revealed that the increase in osteoclasts in OVX' op'op mice is prevented by soluble chimeric VEGFR1 protein or anti'VEGF antibody and by replacement with 17 ' 'estradiol ' Fig. 6 '. These results strongly suggest that the upregulation of VEGF'VEGFR1 in OVX'op'op mouse bone is an important determinant in the increase in the number of osteoclasts by estrogen deficiency. IL'6 may contribute by augmenting the production of VEGF and RANKL. Estrogen deficiency'dependent hypoxia may be involved in upregulation of VEGF expression in bone[54]'. The downregulation of estrogen production leads to hypoxia, a known key regulator of VEGF production[55]', in several organs through decreased nitric oxide synthesis[56]'. The details of mediators in VEGF'dependent osteoclastogenesis are not well established.

Role of VEGF in Endochondral Bone Formation

' VEGF plays an important role in endochondral bone formation. Hypertrophic chondrocytes in the epiphysial growth plate express VEGF[57,58]'. The administration of soluble chimeric VEGFR1 protein completely suppresses blood vessel invasion into the growth plate in mice. Furthermore, the recruitment of osteoclasts and the resorption of terminal chondrocytes are substantially decreased, concomitant with impaired trabecular bone formation[57]'. Mice with the conditional deletion, by means of Cre'loxP'system, of a single VEGF'A allele in collagen type ' ' expressing cells exhibited impaired bone vascularization and mineralization when embryos survive until day E17.5[59]'. A similar phenotype was observed in mice expressing only a single $VEGF_{120}$ isoform ' $VEGF^{120/120}$ [60]'. Although $VEGF^{120/120}$ mice proceed through all stages of embryogenesis, they show skeletal abnormalities including impaired vascularization and deformity of the epiphysial cartilage. Moreover,