A Review of Calcitonin Receptor Expression in Embryonic, Foetal and Adult Tissues, with an Hypothesis on the Connection Between Expression During Foetal Development and Disease

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Abstract: Calcitonin receptor-positive cell populations play a central role in the implantation process of the early blastocyst, in organogenesis of foetal tissues, in several physiological contexts in adult including wound healing and lactation, and in the aetiology of diseases such as certain cancers and cardiovascular disease. Calcitonin receptor (CTR) is expressed by specific precursor cell populations from different lineages including sub-populations of the haematopoietic lineage, as well as differentiated progeny when located in specific micro-environments. Here the central hypothesis is expounded, which proposes that there is the potential of cellular components of the haematopoietic lineage to express CTR, which is a characteristic property.

Keywords: Calcitonin receptor, organogenesis, haematopoiesis.

INTRODUCTION

Calcitonin receptor (CTR) is a member of the G-protein coupled receptor superfamily and functions to bind specific ligands at the cell surface. These signals are amplified through systems, including second messenger systems, and the ligand/receptor complex is internalised in the process. The cycling of the receptor to the cell surface and/or processes of activation may involve receptor activity modifying proteins (RAMPs).

Calcitonin receptor (CTR) and its known cognate ligand (thyro)-calcitonin (CT) have been implicated in calcium homeostasis. The original research papers that described these events were published almost 50 years ago [1-3] and during this period these endocrine functions of CT have had a predominant profile in research on this system. Key cells that express CTR and might mediate such a role have been located in the bone and kidney. The original manuscript described an analysis of the binding of radio-labelled CT to membrane preparations from these tissues [4]. Many subsequent studies have refined these early observations. It is still unclear whether or not other high affinity ligands exist and function in the mammalian system.

In bone CTR is a specific marker of osteoclasts [5-8], in particular osteoclast differentiation [9] and these cells are normally associated with osteolysis. However, in studies using haploinsufficient CTR mice, CTR has been implicated in bone formation [10] although this interpretation is complicated because the genetic background of mice may be a contributing factor [11, 12]. More recently in a global CTR-insufficient strain only a mild increase in bone formation was observed in the basal state [13]. The exact role of CTR is yet to be fully elucidated but it seems to be important for bone and calcium homeostasis under conditions of hypercalcaemia in which osteoclasts and renal cells play key roles.

However, there is also widespread expression of CTR in a range of cell types located in diverse tissues, which suggests serious consideration should be given to the elucidation of further distinct roles and associated mechanisms. In this communication key references and data that highlight this expression are presented and discussed.

Much of the recent research in the identification of CTR-positive cells was made possible with the development of potent anti-CTR antibodies that react with unique epitopes of CTR [14-16], amplified immunohistochemistry and FACS analyses [17]. The identification of the expression of CTR by specific cell types within different biological contexts has led to the formulation of novel hypotheses in regard to the potential for expression and its function by precursor cells and the differentiated progeny. Here the haematopoietic cell lineage is discussed as an example.

THE WIDESPREAD EXPRESSION OF CTR

The Influence of Calcitonin During the Early Foetal Development of Xenopus

Calcium ions play an important role in amphibian cell adhesion and affect the formation of protrusions in amphibian embryonic cells [18], which are considered to play a role in embryonic induction.

Calcitonin influences the early foetal development of Xenopus. The calcitonin-induced distortion of the head and abnormal development in Xenopus embryos is thought to result from inhibition of cell migration (including neural crest cells) into the head region during gastrulation [19]. Its actions in this context may involve the intra-cellular

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1874-3366/09

mobilisation of calcium ions. These observations and interpretations were amongst the earliest in support of a role for the expression of CTR by migrating precursor cells.

CTR Expression During Blastocyst Implantation and Placental Expression in Mammals

Implantation of the mammalian embryo into the uterus requires a complex series of spatio-temporal events that lead to the transformation to a receptive phase, a process in which expression of calcitonin plays a significant role [20, 21]. Concomitantly, CTR mRNA is expressed by the blastocyst between the 1 and 8-cell stages [22]. It is thought that this expression may lead to changes in the calcium activity, which is important for processes in early embryonic life.

Calcitonin was found to be expressed in the mouse maternal trophoblasts [23] of the placenta, and demonstrated more recently in the labyrinthine trophoblasts and intraplacental yolk sac [24]. The receptor CTR is also expressed in the extraplacental yolk sac [24] although its role there is unclear.

The Expression of CTR in the Mammalian Foetus and Postnatal Development

The general pattern of expression of CTR during foetal development was identified with the construction of CTR promoter/reporter gene chimeras in transgenic mouse models. These results were generated from two models in which the reporter gene β-galactosidase was regulated by either the porcine [25] or human [26] promoters of CTR. From these studies it was reported that CTR was expressed at foetal day 15.5 (E15.5) in limb buds, cornea, retina, skin, intercostal muscles, muscles of the face and limbs, the dorsal root ganglia and extensively throughout the CNS [25, 26]. These results suggested the important role that the foetal calcitonin system, in particular CTR, was likely to play in morphogenesis.

A potentially crucial role for CTR expression in foetal development and vitality was further emphasized by the finding that in the CTR-/- homozygote mouse death occurred *in utero* [10].

CTR-Positive (CTR+ve) Precursor Cells that Migrate to Different Organs During Foetal Development

The migration of undifferentiated precursor cells during foetal development is a prominent feature of organogenesis. A well-described example is the migration and role of neural crest cells in the development of many enteric tissues. In parallel with our immunohistochemical studies that mapped the development of CTR+ve neural networks in foetal CNS [14], we identified three potential blast cell populations that expressed CTR. These included populations of neuronal blast cells (CNS, gut, eye), lymphoblasts (gut) and myelo-lymphoid precursors (liver).

CTR-positive neuroblasts (committed neural precursors) have been detected early in the development of the CNS at E12/13 in the anlagen of the hypothalamus and pons [14]. Further evidence of CTR+ve neuronal precursor cells was found with the identification of CTR+ve cells in the region of zones of proliferation (E19) at several locations (subventricular zones) adjacent to the ventricles [14]. The late migration of these CTR+ve precursor cells at E19 is consistent with their commitment to the astroglial lineage although this possibility has yet to be verified [27].

Hemangioblasts are foetal precursor cells that differentiate into progeny of the myeloid, lymphoid and endothelial lineages. They migrate away from the yolk sac early in foetal life [28-31] and occupy a location in the region of the aorta-gonad-mesonephros, before re-locating to the foetal rat liver around E17. Foetal haematopoietic stem cells are located transiently in this compartment [32-34] before migrating to the final (adult) destination in the bone marrow as bone marrow stem cells (BMSCs). Three cell types, which express CTR, were tentatively identified by morphology and location in E17 foetal liver, and these include megakaryocytes [35], Kupffer cells and putative myelo-lymphoid precursors (Fig. **1A-C**). In adult equivalent cell types (eg Kupffer cells, [36]) form part of the same lineage tree (Fig. **2**).

In the Gut what is the Extent of Co-Expression of CTR with the RET Receptor?

The RET (**RE**-arranged during Transfection) receptor tyrosine kinase is encoded by the proto-oncogene *RET* and is activated as the result of the binding of the ligands glial cell-derived neurotrophic factor (GDNF), neurturin, artemin or persephin to the G-protein coupled receptors (GDNF family α receptors, GFRα-1 to 4, respectively).

The origins of the precursors of the putative lymphoblasts located in the lamina propria of gut (Fig. **1D**, [37]) are thought to originate from foetal haematopoietic precursors that have been identified in foetal liver [32-34]. A subset of these precursor cells also express the RET receptor [38]. The GDNF-family of ligands and their receptors are found expressed by adult human immune cells [39].

Our model includes the possibility that some lymphoblasts of gut, which appear to be CTR-positive (Fig. **1D**), are derived from the stem and precursor cells that occupy a niche in the rat foetal liver at E17.

It is of interest that putative neuroblasts in the developing foetal gut also express CTR (Fig. **1D**) and are derived from neural crest cells. Post migration, precursor neural crest cells that eventually differentiate into glial and neuronal cells to form the enteric neural networks, also express the RET receptor [40, 41]. Inactivating mutations of *RET* (as found in Hirschsprung's disease [42, 43]) and the *ret*-/- mouse [44] result in an aganglionic syndrome.

In summary, cell types that express CTR have diverse lineages and their locations are widespread during foetal development. Notably, certain populations (including a sub-population of neural crest cells) of precursors cells are CTR-positive. There is evidence that they also express the RET receptor. Of particular interest here was the observation of the progeny of the myeloid (liver) and lymphoid (gut) lineages that expressed CTR in specific tissues or niches. As discussed below in this article the focus will be on evidence of the expression of CTR in several niches by progeny of the BMSC lineages in adult. This, it is hypothesized, is a fundamental feature of the precursor cells and fully differentiated progeny of the haematopoietic lineage.

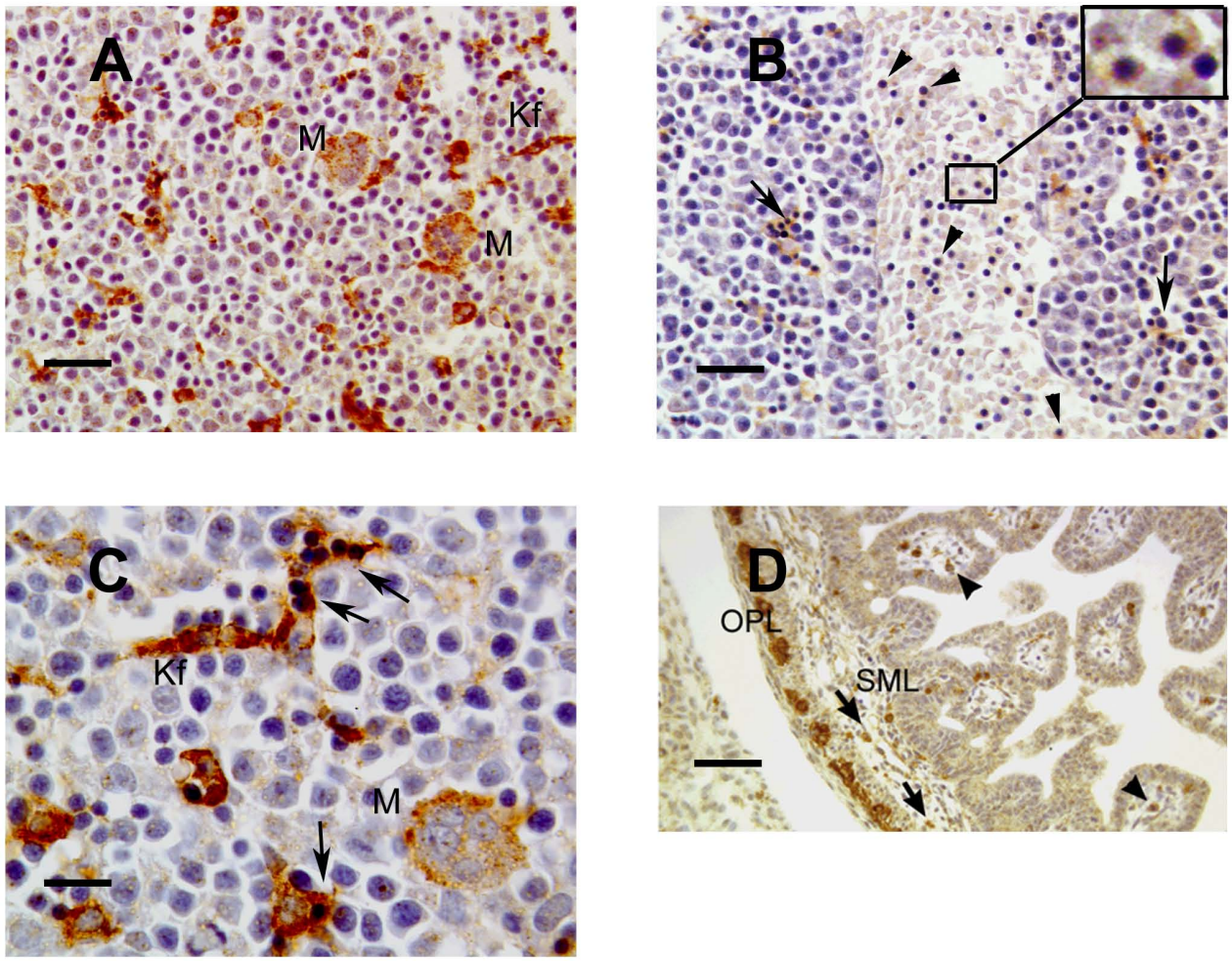

Fig. (1). The expression of CTR by putative precursor cells in the embryo day 17 & 19 (E17 & E19) of rat foetus was identified using immunohistochemistry [DAKO CSA I amplification [14] and the polyclonal antibody anti-CTR antibody (AHP 635, AbD Serotec, UK or 189/10, Welcome Receptor Antibodies, Australia). The specificity of this antibody is discussed in references [14-16, 45, 90]. (**A**) CTR+ve cells in E17 rat liver: megakaryocytes (M) and Kupfer cells (Kf). Bar = 200µm. (**B**) CTR+ve putative myeloblasts (arrows) in E17 rat liver, shown to be present both in the parenchyma and with reduced colour intensity in blood (arrowheads). Two of these CTR+ve cells in blood are shown at higher magnification in the insert. Bar = 200µm. (**C**) Higher magnification showing putative myeloblasts (arrows), megakaryocytes (M) and Kupfer cells (Kf). Bar = 100µm. (**D**) E19 Gut CTR+ve neuroblasts (arrows) and lymphoblasts (arrowheads) in the *lamina propria*. OPL = outer plexiform and SML = sub-mucosal layers. Bar = 400µm.

EXPRESSION OF CTR IN ADULT TISSUES

The expression of CTR by specific cell types has been identified in many tissues. Examples of these include bone [4] and in particular osteoclasts [5, 7, 10, 46], brain [45, 47-49], kidney [4, 15, 50, 51], placenta (mentioned above), testicular cells [25, 52, 53], prostate [54] and breast [55]. Other examples are discussed below.

With the recent availability of high affinity antibodies that specifically detect CTR [14-17, 45, 56] individual cell types that express CTR in normal and diseased tissues have been identified with increasing precision and definition. This precision was not possible using *in vitro* autoradiography with radio-labeled ligands of CTR. Of major interest here is that the precursor cells and differentiating cell types from the BMSC lineage in both normal and diseased tissues may either exhibit transient expression of CTR during migration, healing and development of tissues, or continuous expression, which is often associated with disease.

Cell Types of the BMSC Lineages that Express CTR

In Fig. (**2**) is shown a representation of the lineage tree that has been deduced for bone marrow stem cells and differentiated progeny. Precursor cells (myelomonocytes, CFU intermediates and pro-monocytes) have the potential for limited proliferation as well as commitment to differentiate into specific progeny. These precursor cell populations express CD34 a glycoprotein that is expressed on the cell surface and has been classified as an adhesion molecule. It functions in some cases for the attachment of pro-monocytes to the endothelial surface of blood vessels where they can be induced to differentiate into endothelial cells or foam cells [57]. There is now also evidence (unpublished FACS data) that CTR is expressed by subpopulations of pro-monocytes (CD15, CD33, CD34) and this may determine the destination of the micro-environment into which the cells migrate, and subsequently participate in physiological and pathophysiological events.

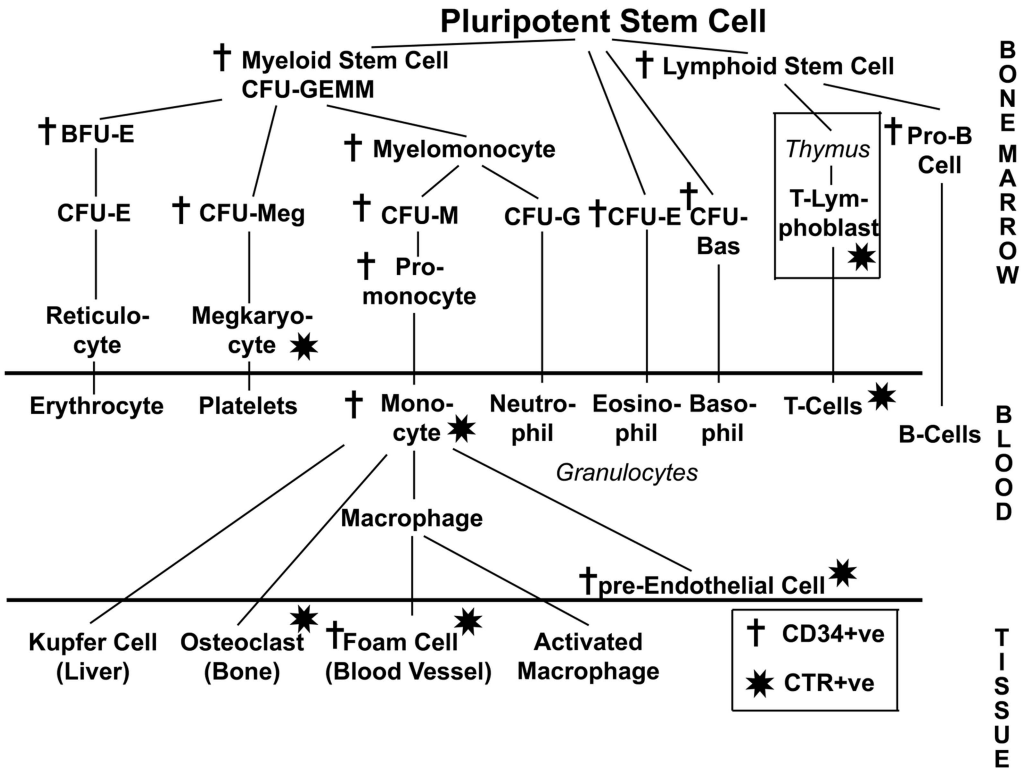

Fig. (2). Haemopoietic lineages that are derived from human myeloid and lymphoid stem cells (bone marrow stem cells or multipotential adult progenitor cells). †CD34-positive cells, *CTR-positive cells.

For instance macrophages, Kupffer cells [36] that line the hepatic sinusoids, foam cells [57] in cardiovascular disease (CVD, discussed further below) and osteoclasts [58] are derived from pro-monocytes of the haematopoietic lineage. In the foetal liver (Fig. **1**) it appears that foetal Kupffer cells exhibit transient expression whereas megakaryocytes may undergo prolonged expression of CTR. A large proportion of megakaryocytes in adult bone marrow are also CTR-positive (data not shown).

T Lymphocytes

CT induces proliferation of T lymphoblasts [59] in rat thymus and presumably these cells express CTR. Normal human T lymphocytes express high affinity CT binding sites [60], a characteristic of CTR. In tonsillar tissue CT is expressed by endothelial cell, which coincides with the migration of T lymphocytes [61]. One interpretation of these coincident events is that CTR constitutes part of a mechanism for the guided recruitment of positive cells into specific sites or micro-environments within target tissues.

Endothelial Cells and Fibroblasts in Healing Wound

A subpopulation of circulating CD34+ cells represents functional endothelial precursors that express VEGFR2 [62]. CT stimulates angiogenesis with HMEC-1 cells that also express CTR [63]. Endothelial cells that line nascent blood vessels and fibroblasts of this granulation tissue of healing wound [at day 7] express CTR compared to normal skin. This expression was completely down-regulated by day 12 when healing was almost complete [56].

It is worth noting that circulating fibrocytes are recruited into skin lesions where they contribute with local fibroblasts (from surrounding tissue) to the healing process [64]. These are likely to be descended from mesenchymal stem cell populations that are unrelated in terms of lineage to the haematopoietic lineage (Fig. **2**).

EXPRESSION OF CTR IN DISEASED TISSUES

Diseased tissues provide a variety of contexts in which to investigate the expression of CTR by particular cell types and an examination of their role in the aetiology of the disease.

Cardiovascular Disease (CVD)

Circulating BMSCs contribute to endothelium of atherosclerotic plaque [65] and neointima [66, 67]. In a rabbit model in which the early events of CVD can be studied such as the invasion of precursor cells into nascent atherosclerotic plaque, several cell types are CTR-positive. These include endothelial cells and foam cells that are derived from blood borne pro-monocytes (mononuclear CD34+), and fibroblasts that probably have originated from mesenchymal stem cells [56, 68]. The expression of CTR is down-regulated as atherosclerotic plaque becomes stabilised [68], which coincides with the end of a healing phase. Such down-regulation was also observed as healing of skin wounds was completed, as discussed above.

In more advanced examples of human CVD, CTR-positive mononuclear cells in the blood have been found associated with the endothelium of diseased human radial and internal mammary arteries [16]. Similar CTR-positive

mononuclear cells have also been noted attached to the endothelium lining of smaller blood vessels in the vasa vasorum of human diseased radial arteries [16]. Furthermore, the expression of CTR in putative tubules that are found in the diseased media of these vessels may represent an intermediate structure for processes that eventually contribute to calcification of the vessel walls [16].

In summary, mononuclear pro-monocytes (CTR+/ CD34+) that have been observed attached to the endothelium and may differentiate into endothelial cells and/or foam cells [57] are recruited into nascent atherosclerotic plaque. These cells and/or differentiated progeny play a role in the early stages of disease in the arterial walls, while CTR-positive cells and structures within the media are also involved later in more advanced CVD [16].

Tumourogenesis

The expansion of malignant tumours from cancer stem cells [69] and migration of other cells are cellular events that may contribute to the formation of solid tumours [70, 71]. These are therefore complex tissues that are comprised of primary tumour cells as well as precursor cells and other cells recruited into the tumour. These events appear to share some features with the paradigms and processes of foetal organogenesis including the advent of zones of proliferation and the recruitment of migrating precursor cell populations, the role of growth factors and vasculogenesis, albeit with an altered morphological order.

Primary tumour cells from breast [55, 72] and prostate [73] cancers, and interestingly, malignant plasma cells from multiple myeloma [17] express CTR.

Of note, many isolated cell lines derived from tumours have been described that express CTR. A short list of those that have CT binding sites or express CTR includes osteoclastoma [74] and central giant cell granuloma cells [75, 76], breast cancer-related cell lines MCF-7 [77] and T47D [78], an ovarian carcinoma cell line [79], thyrotrophs [80], and leukaemic blast cell lines express CT binding sites [81, 82], CTR mRNA [17, 83] and CTR protein detected by FACS analysis (unpublished data, [17]). However, it is not clear what part the primary cells that gave rise to these cell lines, might have played in tumourogenesis.

What is interesting is the finding of CTR expression by primary leukaemic blast cells. We reported last year [56, 84] on our finding of CTR expression by primary CD34+ blast cells from the bone marrows (BM) of cohorts of patients diagnosed with acute myeloblastic or lymphoblastic leukaemia (AML , ALL). In the BM aspirants of these patients we discovered significant populations of CD34+/CTR+ blast cells. In leukaemia expansion of blast populations can reach crisis point for patients and this event is central to the aetiology of the disease and outcomes for patients. More recently, the co-expression of CTR and CD117, a marker associated with normal precursor cells of the haematopoietic lineage has been identified (unpublished observations).

These findings are of interest because they demonstrate that CTR is expressed by some primary tumour blast cells that may represent the underlying cause of the disease, and in the case of multiple myeloma, contribute to the clinical manifestations such as osteolysis [17]. A role for the expression of CTR by these blast cells is yet to be demonstrated but may involve mechanisms for the compartmentalisation of these undifferentiated cells in bone marrow. Spill-over into the peripheral blood occurs when these blast cell populations expand unchecked. In ALL and AML CTR may be expressed by cells of the haematopoietic lineage (Fig. **2**) as an incidental marker rather than being strictly oncogenic.

These data suggest that CTR may be expressed in two phases in relation to lineage restriction. The first corresponds to precursor or blast cell stages within bone marrow that may spill over into blood as disease progresses. As progeny mature and become integrated into target tissues there may be a second phase that is determined largely by the local tissue micro-environment. Such biphasic expression has previously been noted in the developing kidney [15]. The finding of CTR expression by primary plasma cells capable of osteolysis from patients with multiple myeloma [17] may represent a version of the second phase but expression may be independent of the tissue micro-environment.

The potential to express CTR by some of the terminal differentiated progeny as shown in the lineage tree is yet to be described, such as expression by granulocytes (eosinophils, neutrophils and basophils) although we have anecdotal evidence from observations in human tissues subjected to immunohistochemical analysis that the former two do express CTR.

In summary of this section, the hypothesis states that the potential expression of CTR is a basic characteristic associated with lineages within this haematopoeitic system, and CTR is expressed in two distinct phases, one associated with precursor populations of BMSCs, and the second with recruitment differentiated progeny into target tissues.

The Expression of the RET Receptor (Proto-Oncogene Product) in Normal Bone Marrow and Leukaemia

In the BM environment where haematopoiesis is normally tightly regulated there is thought to be an interaction between stromal cells and RET-positive haematopoietic blast cells, which plays a role in the regulation of the differentiation of myelomonocyte precursors and T-cells [85, 86]. RET expression was also detected in B-cells and monocytes [39]. Important for this discussion, *RET* expression is up-regulated by leukaemic blast cells but confined to the myeloid lineage (maximal in intermediate phenotypes) in AML. Given the known properties of the RET receptor as an oncogene product it is somewhat surprising that mutations in the *RET* gene have not (yet) been associated with some form of leukaemia [87]. A possible explanation is that up-regulation of RET receptor may be secondary in these tumourogenic events and activation may result from activation by ligands of the GDNF family.

In summary, it appears that expression of RET receptor is confined to a limited number (subset) of cell intermediates and progeny of the haematopoietic lineage tree in which there is also expression of CTR in many cases.

Possible Cellular Mechanisms Involving CTR

i). Retardation of the Cell Cycle

A calcitonin response element (Sp1 binding site) was identified in the promoter of the human $p21^{WAF1/CIP1}$ gene encoding a cyclin-dependent kinase inhibitor [88]. Calcitonin induced cell cycle arrest at the G_2/M phase in cells transfected to express the insert-negative isoform of CTR [89]. Such a mechanism may be relevant to CTR-positive precursor cells, including neuroblasts [14], precursors of the haematopoietic lineages and quiescent satellite stem cells associated with muscle [90]. Such a mechanism may be one important control mechanism for haematopoiesis and CTR may be down-regulated or inactivated during leukaemogenesis.

It is yet to be established whether such mechanisms also play a role in the control of cellular proliferation during wound healing and tubulogenesis in the developing kidney.

ii). Migration and Recruitment of Precursor Cells and/or Progeny

There are several instances and reports that provide evidence for the involvement of CTR in mechanisms of cell migration. For instance, immature monocytes mobilised in blood are CD34+/CTR+ and attach to the endothelial layer of diseased blood vessels (CVD, [16]). Second, CT promotes the invasiveness of prostate cancer cells that express CTR [91]. Third, endothelial cells that line blood vessels of the tonsils express CT and promote the migration of lymphocytes [61].

iii). Promotion of Differentiation, for Instance Progeny of the Haematopoietic Lineage

The expression of CTR is thought an important factor in the control of the terminal differentiation of osteoclasts (derived from monocytes) and osteolysis [6, 10, 46, 92, 93]. For other progeny of the same haematopoietic lineage that express CTR, such as lymphocytes, megakaryocytes, foam and endothelial cells, CTR may also play a role in the terminal differentiation process.

Micro-Environment for CTR+ve Cell Types

The induction of CTR expression may be dependent on cognate ligands and/or other factors within a defined micro-environment as well as the lineage of the recruited cells. Such a phenomenon would correspond to the second phase of CTR expression discussed above. In the foetus such niches are evident from the expression of CTR by hemangioblasts in liver and later in bone marrow. It remains to be tested whether hemangioblasts of the AGM region and earlier in the yolk sac, also express CTR.

In adult, within the bone and the BM niches, it is well known that endogenous cells provide essential growth and survival factors for instance stromal cell-derived factor (SDF-1, CXC-12), RANKL, GDNF and many other factors. These factors contribute to the tight regulation of subpopulation sizes of specific cell types and haematopoiesis in general.

During the expansion of the CTR+ve BM populations in diseases such as leukaemia and in an inflammatory response including wound healing and CVD, spill-over of blast cells into peripheral blood appears to be a common feature. In instances when CTR functions in migration it is likely that the cognate high affinity ligand for CTR is synthesized within the target tissues (such as CVD [16] and tonsils [61]) that recruit CTR-positive cells.

It is proposed that within the micro-environment of atherosclerotic plaque such ligands are expressed and that CTR contributes to a homing mechanism for precursor cells recruited into these tissues. These events are important for our understanding of the principles of healing in diseased vessels and in the potential treatment of CVD that is a high risk factor for stroke.

Hypothesis on the Functional Role of CTR

The hypothesis states that CTR functions to reduce or stall the cell cycle of precursor cells prior to migration from the initial micro-environment of bone marrow and therefore contributes to the control of cell population sizes. The apparent reduced expression of cells as they migrate away from the foetal liver (Fig. **1**) is consistent with this idea.

The second period of expression is induced as cells are recruited into the target micro-environment such as diseased vessels, as part of the homing mechanism. One prediction is that the ligand for CTR should be expressed in the target tissue.

SUMMARY

CTR is expressed by a majority of cell types both precursor cells and their differentiated progeny associated with the haematopoietic lineage, and expression may be separated into two phases. In some tissues undergoing normal physiological functions such as wound healing and CVD as discussed above, the expression of CTR may be part of a mechanism involved in the recruitment and migration of blood-borne precursors important for subsequent healing or that play a significant role in the aetiology of more advanced disease.

The expression of the RET receptor appears to overlap that of CTR in a spatio-temporal sense in some branches of the lineage tree but they may function independently of each other. As noted above these two membrane proteins are co-expressed in other foetal cell populations such as neural precursors in the enteric nervous system that are derived from post-migratory neural crest cells. It remains to be determined what significance co-expression might have for organogenesis.

It will be interesting to identify the putative high affinity ligands for CTR, which may be expressed in different target tissues in mammals and that promote the recruitment of CTR-positive precursor cells.

Finally, transient expression of CTR mRNA and/or protein may be an important mechanism for the control of physiological events such as renal development [15], wound healing and T-cell migration [61]. On the other hand, progression to advanced CVD and cancer may be accompanied by up-regulated and prolonged expression of CTR by specific cell types in diseased tissues.

ACKNOWLEDGEMENT

The advice and contributions of Professor John Furness (University of Melbourne) in relation to the original analysis

of immunohistochemical data generated from the rat foetal studies are gratefully acknowledged. He also read the manuscript prior to publication.

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Received: January 09, 2009 Revised: March 02, 2009 Accepted: April 15, 2009