Expression profile of cell adhesion molecules in the vascular wall of transgenic mice overexpressing calreticulin

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Abstract

Calreticulin (CRT) is an endoplasmic reticulum resident protein that fulfills many functions, including acting as a chaperone and regulating intracellular Ca^{2+} homeostasis. CRT has been shown to be essential for cardiac development, and has been implicated in the regulation of cellular adhesion. Recently, a transgenic mouse that overexpresses CRT in vascular smooth muscle cells (*SMCRT*) has been developed in our laboratory. These mice develop hemangioendothelioma, an aggressive type of vascular tumour that also occurs in humans. We hypothesized that changes in cell adhesion and migration induced by CRT overexpression contribute to the development of these tumours. The aim of this study was to examine the expression and localization of several cell adhesion proteins in vascular smooth muscle cells isolated from the *SMCRT* mice. Western blot, immunohistochemical and immunocytochemical analyses with specific antibodies to detect the different adhesion molecules were used. Our results show that there is a significant decrease in both vinculin and VCAM-1 expression levels in the *SMCRT* cells as compared to the wild type cells. Furthermore, the expression of N-Cadherin, focal adhesion kinase and laminin did not change. The changes in vinculin and VCAM-1 expression might be due to the chaperone role CRT plays in the folding and maturation of newly synthesized proteins. However, CRT as a regulator of intracellular Ca^{2+} homeostasis may also affect the rate of transcription of these genes.

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Introduction

The SMCRT mouse

A transgenic mouse has been developed in our laboratory that overexpresses calreticulin (CRT) exclusively in vascular smooth muscle cells (*SMCRT* mice). This tissue specific expression was accomplished by cloning mouse CRT cDNA downstream of a truncated SM22α promoter (Li *et al.,* 1997). These mice serve as a novel mouse model for determining the role of CRT in vascular development and function. The major phenotypic defect in the *SMCRT* mice is the development of large hemagioendotheliomas in the thorax that originate mainly from the aorta. Other phenotypes of these mice include the development of hemangioma in the skin, liver, lung, and kidney, increased angiogenesis, as well as heart and kidney failure.

Calreticulin

CRT was first isolated and purified based on its high affinity for calcium in 1974 by Ostwald and Maclennan. It is 70% identical at the nucleotide level between humans and mice, and is comprised of 9 exons and 8 introns (Fliegel *et al.,* 1989b; Smith *et al.,* 1989). The CRT protein is made up of 400 amino acids and has a calculated molecular weight of 46 kDa, but runs at ~55-60 kDa on SDS-PAGE due to its high acidic residue content at its C-terminal end (Coppolino *et al.,* 1998; Matsuoka *et al.,* 1994; Smith *et al.,* 1989). At the amino acid level, CRT is highly conserved between human, rabbit, rat and mouse, displaying 90% between-species conservation (Coppolino *et al.,* 1998; Michalak *et al.,* 1999). The protein is comprised of three distinct domains (Coppolino *et al.,* 1998; Fliegel *et al.,* 1989b; Michalak *et al.,* 2002). The N-domain is hydrophobic and contains three cysteine residues, two of which form intramolecular disulphide bridges which force

this domain to adopt a globular conformation (Coppolino *et al.,* 1998; Fliegel *et al.,* 1989b; Matsuoka *et al.,* 1994; Michalak *et al.,* 2002). This region is quite similar to calnexin, and may be involved in glucose binding (Baksh *et al.,* 1995). A proline-rich P-domain contains a high-affinity, low-capacity calcium binding region (Baksh *et al.,* 1991; Coppolino *et al.,* 1998). Two anti-parallel β-sheets in the P-domain form a hairpin fold that connects it to the N-domain (Ellgaard *et al.,* 2001). The P and N-domains are thought to be responsible for the chaperone function of CRT (Michalak *et al.,* 2002). The C domain of CRT contains a high-capacity, low-affinity calcium binding site (Baksh *et al.,* 1991; Coppolino *et al.,* 1998; Fliegel *et al.,* 1989b; Michalak *et al.,* 2002). A highly acidic region within the C-domain, caused by a high concentration of negatively charged aspartate and glutamate amino acid residues, is thought to bind calcium (Baksh *et al.,* 1991; Coppolino *et al.,* 1998; Fliegel *et al.,* 1989b; Smith *et al.,* 1989). The primary structure of this domain is currently unknown, but may be in a linear conformation caused by the charge repulsion from the acidic residues (Fliegel *et al.,* 1989b). The extreme C-terminal end of CRT contains a KDEL endoplasmic reticulum (ER)-retention sequence that resequesters the protein to the ER lumen from the golgi apparatus (Coppolino *et al.,* 1998; Fliegel *et al.,* 1989b; Michalak *et al.,* 1999; Smith *et al.,* 1989).

Localization and funtion

Ubiquitously expressed in most organs, CRT is sequestered to the ER in non-muscle and the sarcoplasmic reticulum in skeletal and cardiac muscle by its ER retention sequence (Fliegel *et al.,* 1989a; Sontheimer *et al.,* 1995). CRT fulfils various roles, but primarily acts as I) a chaperone and II) a regulator of intracellular calcium homeostasis (Michalak *et al.,* 1999). Chaperone proteins are important residents of the ER lumen, as

they bind to newly synthesized proteins and aid them in folding into their correct tertiary structure. In addition, chaperone proteins also inhibit the aggregation of misfolded proteins within the ER. The chaperone nature of CRT has been demonstrated by its preference for binding to immature versus mature protein conformations (Jorgensen *et al.,* 2000; McDonnell *et al.,* 1996; Saito *et al.,* 1999). CRT has previously been shown to act as a chaperone for several proteins, including laminin (McDonnell *et al.,* 1996), IgY antibody (Saito *et al.,* 1999), citrate synthase (Saito *et al.,* 1999), malate dehydrogenase (Saito *et al.,* 1999), ovalbumin (Jorgensen *et al.,* 2000), HIV-1 envelope glycoprotein (Otteken *et al.,* 1996), myeloperoxidase (Nauseef *et al.,* 1995) and vinculin (Opas *et al.,* 1996).

Calcium homeostasis is very important within the cell. Cytoplasmic calcium levels are responsible for controlling such processes as cell motility, proliferation, contraction, signal transduction and gene expression (Meldolesi *et al.,* 1998). There are three main factors that regulate intracellular calcium pools. These include the SERCA pump, responsible for the reuptake of calcium into the ER lumen, the inositol triphosphate (IP_3) receptors that control release of calcium from the ER and ER calcium binding proteins (Meldolesi *et al.,* 1998). Down regulation of CRT in NG-108t5 cells using anti-sense oligo caused a significant decrease in ER calcium storage (Liu *et al.,* 1994), while over-expression of CRT in Hela and mouse L fibroblasts increased the ER calcium store (Bastianutto *et al.,* 1995; Mery *et al.,* 1996). Additionally, there is evidence that CRT may regulate IP_3 receptor activity, thus affecting the quick release of calcium into the cytoplasm (John *et al.,* 1998; Liu *et al.,* 1994; Michikawa *et al.,* 1994). These data clearly demonstrate CRT's involvement in calcium homeostasis within the cell.

In addition to having a role as a chaperone and being involved in calcium homeostasis, CRT has also been shown to modulate new blood vessel growth (angiogenesis). The activity of CRT promoter has been detected early in development within the heart, somitic vessels, the descending aorta, as well as many smaller arteries, thus suggesting a role for CRT in the early regulation of blood vessel formation in the mouse (Mesaeli *et al.,* 1999). CRT is downregulated after birth in the heart, but expression is maintained in blood vessels throughout life, in both adult endothelial and vascular smooth muscle cells (Mesaeli *et al.,* 1999; Milner *et al.,* 1991; Patton *et al.,* 1995; Tharin *et al.,* 1992). Exogenous CRT has been shown to decrease intimal hyperplasia following balloon angioplasty (Kuwabara *et al.,* 1995), and both endothelial proliferation and tumour angiogenesis in Burkitt lymphoma have been shown to be inhibited by either CRT or vasostatin, a molecule identical to the N-domain of CRT (Pike *et al.,* 1998; Pike *et al.,* 1999; Yao *et al.,* 2002). Vasostatin selectively inhibits tumour angiogenesis without affecting angiogenesis associated with wound healing (Lange-Asschenfeldt *et al.,* 2001; Yao *et al.,* 2002). Vasostatin treated tumour cells had a higher rate of apoptosis, indicating a decrease in tumour progression (Xiao *et al.,* 2002). CRT's abilitiy to increase nitric oxide production and decrease MMP-2 activity, which is responsible for extra-cellular matrix degradation during angiogenesis, are both mechanisms by which CRT may regulate angiogenesis (Ito *et al.,* 2001).

As well as being expressed in the developing and adult vasculature of the mouse, CRT is also highly expressed in the embryonic heart, suggesting a role for CRT in cardiac development (Mesaeli *et al.,* 1999). This hypothesis is supported by several pieces of evidence. Deleting the CRT gene in mice results in embryonic death, since no

crt -/- mice are born live (Mesaeli *et al.,* 1999; Rauch *et al.,* 2000). Conversely, mice over-expressing CRT in cardiomyocytes experience stunted growth and have complete heart block resulting in sudden death (Nakamura *et al.,* 2001).

Cell adhesion

The formation of distinct tissues and their organization into functional organs is dependent on adhesive interactions between cell adhesion molecules (CAMs) and between CAMs and the extra cellular matrix (ECM) (Lodish *et al.,* 2004). CAMs mediate both cell-cell and cell-ECM interactions (Lodish *et al.,* 2004). These interactions are essential for the transfer of information between cells, and communication between the cell and its surroundings, as well as regulating activities such as cytoskeletal organization, cell motility and migration, cell proliferation, survival, growth and differentiation (Hynes 1999; Karp 2002; Lodish *et al.,* 2004). CAMs may mediate interactions between cells of the same type (homotypic adhesion), or between cells of different types (heterotypic adhesion) (Lodish *et al.,* 2004). They may either be broadly distributed in a cell, or occur in specific clusters, forming cell-cell junctions (Lodish *et al.,* 2004). Four families of CAMs exist: the cadherins the immunoglobin family, the integrins and the selectins. The cadherins mediate calcium dependent cell-cell homophilic interactions. The immunoglobin family contains multiple immunoglobin domains, and are components of the tight junction that mediate calcium independent cell-cell interactions. The integrins are present in the form of a heterodimer, are transmembrane proteins that mediate adhesion primarily to the ECM. Finally, selectins function in interactions between endothelial cells and leukocytes, and mediate calcium dependent interactions with glycoproteins and glycolipids on the surface of neighboring cells (Hynes

1999; Lodish *et al.,* 2004). Adaptor proteins are present on the cytosolic side of many cell junctions and function to link the CAM to the cytoskeleton. These adaptor proteins recruit intracellular molecules to act in cell signaling pathways, which in turn may control protein activity and gene expression within a cell (Lodish *et al.,* 2004). Adhesion receptors bear a close resemblance to growth factors in how they signal within a cell, and should be considered alongside growth factors (Hynes 1999).

Cellular adhesion is particularly important within the vascular wall since changes in CAM profile occur during vascular repair as well as during angiogenesis (Moiseeva 2001). ECM characteristics and adhesion receptors play a large role in regulating vascular cell adhesion (Moiseeva 2001). The ECM is a dynamic structure, constantly being remodeled, and is made up of collagens, protein fibers and matrix proteins which provide mechanical support to the overlying tissue (Lodish *et al.,* 2004). Receptors found on the ECM are bound by cellular CAMs, providing a link between the cytoskeleton and the ECM (Moiseeva 2001). The ECM also seems to play an important role in regulating vascular smooth muscle phenotype, with matrix-cell communication occurring through CAMs (Moiseeva 2001). The integrin family of CAMs provides the primary link between the cell and the ECM receptors. The integrins are bound cytoplasmically to cytoskeletal proteins such as talin, vinculin, α-actinin and filamin (Moiseeva 2001). Integrin-ligand interactions are responsible for maintaining smooth muscle phenotype, with binding being tightly regulated via conformational changes due to cell signaling (Moiseeva 2001). Intercellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) have also been detected in vascular smooth muscle (Moiseeva 2001). These two members of the immunoglobin-like CAM family are likely to be involved in

inflammatory and immune processes by enabling leukocyte trafficking within vascular tissue through interactions with leukocyte integrins (Moiseeva 2001). There is also evidence that the immunoglobin family receptors make cytoskeletal connections much like the integrins and cadherins, making the immunoglobin family one of great diversity (Hynes 1999). Cadherins are involved in β-catenin mediated signaling, and have several functions within the vascular wall (Moiseeva 2001). E-cadherin has been shown to be upregulated upon vascular injury, coinciding with the beginning of smooth muscle proliferation (Moiseeva 2001). N-cadherin seems to mediate endothelial-smooth muscle cell interactions by means of contact inhibition of growth (Moiseeva 2001).

Calreticulin in cell adhesion

CRT has been shown to modulate cell adhesion (Coppolino *et al.,* 1997; Coppolino *et al.,* 1998; Fadel *et al.,* 1999; Fadel *et al.,* 2001; Michalak *et al.,* 1999; Opas *et al.,* 1996; Zhu *et al.,* 1997). The earliest evidence that CRT may be involved in cell adhesion was that CRT binds to the tail of α-integrin *in vitro*, modulating its function (Coppolino *et al.,* 1997; Coppolino *et al.,* 1998; Leung-Hagesteijn *et al.,* 1994). For CRT to bind to α-integrin it would be necessary that CRT be found in the cytoplasm. However, CRT is localized to the ER, suggesting that any changes in cell adhesion by CRT must be indirect (Fadel *et al.,* 1999; Opas *et al.,* 1996). Proposed mechanisms for CRT's influence on cell adhesion indirectly from the ER include modulating gene transcription of adhesion molecules (Fadel *et al.,* 1999; Opas *et al.,* 1996), changing integrin-dependent calcium signaling (Coppolino *et al.,* 1997), and most recently evidence suggests that CRT alters protein-tyrosine kinases and/or phosphatases, which can affect protein phosphorylation/dephosphorylation (Fadel *et al.,* 2001; Michalak *et al.,* 1999). The

phosphorylation of β-catenin, a structural component of cadherin-dependent adhesion complexes, is altered by changes in CRT levels (Fadel *et al.,* 2001). Overexpression of CRT tends to increase cell adhesion, whereas decreases in CRT resulted in a decrease in cell adhesion (Fadel *et al.,* 1999; Opas *et al.,* 1996). To date, CRT has been shown to alter levels of vinculin and N-cadherin (Fadel *et al.,* 1999; Fadel *et al.,* 2001; Opas *et al.,* 1996). CRT overexpression in retinal pigment epithelial cells increases vinculin levels (Fadel *et al.,* 1999), whereas overexpression of CRT in L fibroblasts increases both vinculin and N-cadherin levels (Fadel *et al.,* 2001).

Hemangioendothelioma

Hemangioma is caused by abnormal vascular development. There are many types of this pathology, which are mainly benign. *SMCRT* mice develop hemangioma on the skin and some internal organs. The hemangiomas that develop in *SMCRT* mice originate at the base of the aorta. The earlisest that a hemangioma developed in these mice was three months post nataly. The hemangioendothelioma seen in *SMCRT* mice are similar to human Kasabach-Merritt Syndrome (KMS). Symptoms of KMS include giant hemangioma with consumptive coagulopathy, disseminated intravascular coagulation, and congestive heart failure. Hemangiomas, when they develop in the thorax, cause airway compression and respiratory distress, high-output cardiac failure, and when present in conjunction with KMS, may be life-threatening (Maguiness *et al.,* 2002). KMS is associated with both Kaposiform hemangioendothelioma and tufted angiomas (Enjolras *et al.,* 1997; Maguiness *et al.,* 2002). Tufted angiomas are benign, slow-growing angiomas occurring in the skin and subcutaneous tissue of young patients (Jones *et al.,* 1989). Kaposiform hemangioendotheliomas are aggressive vascular tumours which may

occur in soft tissue of the trunk, extremities and retroperitoneum (Gampper *et al.,* 2002). The Kaposiform hemangioendothelioma is characterized by a thin-walled vessel filled with sheets and lobules of round and spindle-shaped endothelial cells, erythrocytes and thrombus, and have been defined as having an "intermediate" or a "borderline" malignancy potential (Maguiness *et al.,* 2002; Perkins *et al.,* 1996). However, they do not metastisize (Maguiness *et al.,* 2002). This vascular tumour has a high mortality rate, primarily due to inadequate treatment options (Maguiness *et al.,* 2002; Powell 1999). Histological analysis of the hemangioendotheliomas isolated from the *SMCRT* mice showed a vessel-like structure with an outer layer of what appears to be endothelial cells. Within the tumours are several lumen-like structures that also seem to be lined with endothelial cells. Found within the tumour were blood cells and cells that resembled both epithelial cells and mesenchymal cells.

Aim and hypothesis

The aim of this study was to determine the expression and localization of cell adhesion proteins in vascular smooth muscle cells isolated from the SCMRT transgenic mice. This would give insight into *in vivo* changes in cell adhesion protein expression mediated by increased CRT levels, which have previously been studied solely *in vitro*. Secondly, it investigated a possible mechanism that controls the development of vascular hemangioendothelioma. The mechanism for development of Kaposiform hemangioendothelima is currently unknown (Maguiness *et al.,* 2002). Previous studies have shown that cell adhesion plays a critical role in tumour development, proliferation and migration (Gassmann *et al.,* 2004; Playford *et al.,* 2004; Reynolds *et al.,* 2004).We hypothesize that changes in cell adhesion and migration of the vascular smooth muscle

cells due to increased intracellular CRT levels contribute to the development of hemangioendothelioma. Understanding the root molecular cause of these tumours will allow for the design of novel therapeutic approaches.

Methods

Stable Cell Lines

Stable cell lines were established from *SMCRT* mice as well as from embryos that were homozygous knock outs for the CRT gene (*crt-/-*). Wild type control cell lines for both cell types were established. These cell lines were used to examine the effects of both gain as well as loss of CRT function in the cell. Vascular smooth muscle cells isolated from *SMCRT* mice were used to investigate the effects of CRT overexpression, while mouse embryonic fibroblasts (MEFs) isolated from the *crt-/-* mice were used to study the loss of CRT function. MEFs were used rather than smooth muscle cells due to the early embryonic lethality of *crt-/-* mice.

Stable Cell Line Generation

Mouse Embryonic Fibroblasts

Pregnant mice were sacrificed at 14 days gestation and the embryos dissected. A portion of the embryo was taken for genotyping. The remainder of the embryo (excluding the head and heart) was rinsed in sterile 1X PBS and cut into small pieces which were then placed in a sterile tube along with 500µl of Trypsin (0.05% in PBS). The tube was then rotated at 37°C for five minutes. Cells were dispersed using a sterile bulb pipette. Avoiding larger pieces, the solution was transferred to a small culture dish with Dulbecco's Modified Eagle's Medium (DMEM) containing 20% FBS and L-glutamine. Pieces of tissue were transferred to a new plate after three days, and cells migrating out were passaged. Cells were then transfected with a plasmid containing the SV40 large T antigen, which results in an immortal cell line. Two weeks after trasfection, cells formed

colonies. These colonies were isolated and sub-cultured and were the basis of the stable cell lines.

Smooth Muscle Cells

Adult wild type (*wt*) and transgenic mice overexpressing CRT in vascular smooth muscle cells (*SMCRT*) were sacrificed and their descending aortas dissected out. Harvested descending aortas were then cleaned of surrounding fat and connective tissue before being washed several times in sterile PBS. The descending aortas were then cut into fine pieces and placed in a 3cm Petri dish with DMEM containing 20% FBS and L-glutamine. Cells were allowed to grow out from the tissue pieces (explant culture), and were subsequently subcultured to a new plate and transfected with a plasmid containing the SV40 Large T antigen. Transfected cells were allowed to form colonies, which were then screened for smooth muscle versus fibroblast content by immunocytochemical staining for smooth muscle actin. The purest colonies (greatest percentage of vascular SMCs) were then selected and expanded.

Cell culture and lysate preparation

Wild-type (*wt*) and CRT deficient (*crt-/-*) mouse embryonic fibroblasts cells (MEFs) were cultured in DMEM containing 10% fetal bovine serum (FBS) and 0.5% penicillin-streptomycin. Wild-type smooth muscle (*wtSMC*) and smooth muscle cells over-expressing CRT (*SMCRT*) were maintained in DMEM with 5% FBS and 0.5% penicillin-streptomycin. Cells to be lysed were washed in cold PBS before incubation in 2.5% PMSF and 0.25% cocktail of protease inhibitors diluted in New RIPA buffer for five minutes. Cells were then collected and spun at 7000 RPM for five minutes at 4°C. Supernatant was collected and stored at -20°C until use.

Protein assays using Bio Rad protein assay reagents and 96-well plates were carried out in order to determine the protein concentrations. To each well, 25µl of reagent A' was added, followed by 5µl of the protein sample. Two hundred µl of reagent B was then added and allowed to incubate at room temperature for 15 minutes. Resultant optical densities were then read by a Dynex MRXtc Revelation microplate reader and corresponding protein concentrations determined by comparison with optical densities of known standards.

Western Blotting

Proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels. Twenty to 30µg of protein were loaded per lane. Following separation, the proteins were transferred to a nitrocellulose membrane using a wet transfer system at 4°C for two hours with a current of 350mA. Membranes were blocked for 20 minutes with 5% milk powder diluted in 1X PBS. Following blocking, membranes were incubated for one hour at room temperature with one of the following primary antibodies diluted in 1% milk powder in PBS: mouse anti-vinculin (1:300 Sigma), rabbit anti-focal adhesion kinase (FAK) (1:300 Santa Cruz), rabbit anti-laminin (1:200 Sigma), rabbit anti-N-cadherin (1:200 Santa Cruz), or goat anti-vascular cell adhesion molecule-1 (VCAM-1) (1:200 Santa Cruz). Membranes were then incubated at room temperature for one hour using the appropriate horseradish peroxidase (HRP) conjugated secondary antibody at a dilution of 1:10,000. An enhanced chemiluminescence detection system (Western Blot Luminol Reagent, Santa Cruz) and a Fluor-S Max MultiImager (Bio Rad) were used to detect the protein bands. Membranes were washed with 1X PBS plus 0.05% TWEEN and stored in 1 X PBS overnight at 4°C.

The following day, membranes were probed with a rabbit anti-actin (1:1,000) primary antibody and an HRP labeled secondary antibody (1:10,000), each for one hour and visualized as described above. Actin served as a loading control to normalize for differences in loading. Protein bands were quantified via densitometry using Bio Rad's Quantity One software.

Immunohistochemistry

Immunohistochemistry was carried out in order to localize cell adhesion molecule expression within the vascular wall. Descending aortas were fixed in 4% paraformaldehyde in PBS for one hour at room temperature before being embedded and frozen in Tissue Tek's O.C.T compound. They were then sectioned in cross section at 7μm using a Shandon 0620 cryotome at -22˚C. Sections were mounted on Vecta Bond (Vector) coated slides. Sections were then exposed to UV radiation for fifteen minutes in order to reduce (photo-bleach) auto-fluorescence caused by the elastin fibers. Following a five minute wash in 1X PBS, sections were blocked for 20 minutes using Roche Blocking Reagent (1%). Vector's avidin/biotin blocking kit was then applied for 30 minutes (as per included instructions). Slides were washed in 1X PBS and then stained with primary antibody diluted in Roche Blocking Reagent (1%) for one hour at room temperature [goat anti-VCAM-1 (1:100 Santa Cruz), rat anti-PECAM (1:150 BD Biosciences), goat anti-integrin α_v (1:100 Santa Cruz), goat anti-integrin β_3 (1:100 Santa Cruz), rabbit anti-FAK (1:100 Santa Cruz), rabbit anti-HA (1:300 Sigma), or mouse anti-smooth muscle actin (1:400 Sigma)]. An appropriate biotinylated secondary antibody (1:200, diluted in Roche Blocking Reagent) was then applied for one hour at room temperature, followed by a five minute wash with 1X PBS. Sections were then incubated with strepavidin-conjugated

Texas Red (1:70 Jackson Laboratories), and washed in 1X PBS for five minutes. For detecting smooth muscle actin, sections were incubated with a FITC-conjugated secondary antibody (1:70 Jackson Laboratories). Molecular Probes' SloFade anti-fade kit with DAPI (nuclear stain) was then applied prior to the addition of a coverslip. Coverslips were adhered to the slide using nail polish. Images were captured using a Zeiss Axiovert Fluorescent microscope.

Immunocytochemistry

Immunocytochemistry allows for localization of cell adhesion molecules within the cell. All four cell types were plated on glass coverslips and fixed in 4% formaldehyde (in PBS) for fifteen minutes at room temperature. Coverslips were then washed with 1% saponin in PBS three times and blocked with 2% milk powder and 0.1% saponin in PBS for 30 minutes in a holding container. Cells were incubated with primary antibody for one hour at room temperature [goat anti-VCAM-1 (1:100 Santa Cruz]. An anti-goat secondary FITC-labeled antibody was then applied for one hour (1:70 Jackson Laboratories). Actin filaments were stained using Texas Red-labeled phalloidin toxin (Molecular Probes). When probing for vinculin (1:50 Sigma), cells were fixed for two minutes in a 50:50 methanol and acetone mixture. Coverslips were mounted on slides using Molecular Probes' SloFade anti-fade kit with DAPI. Nail polish was used to fix the coverslip to the slide. Images were captured using a Zeiss Axiovert Fluorescent microscope.

Results

Most proteins involved in cell adhesion are synthesized in the ER where their folding is regulated by ER chaperones. CRT is an ER chaperone which has been shown to affect cell adhesion. To examine the changes in levels of cell adhesion proteins with differential expression of CRT, western blot analyses were carried out using specific antibodies. Figure 1 shows representative western blots demonstrating changes in vinculin and VCAM-1 expression with altered CRT expression. CRT deletion (*crt-/-* MEF) resulted in a 123% increase in vinculin level (Figure 2), which was statistically significant ($p<0.05$) as compared to *wt* MEF cells. Overexpression of CRT in *SMCRT* on the other hand, resulted in a significant decrease (37% with $p<0.05$) in vinculin level (Figure 2). Interestingly, both *crt-/-* and *SMCRT* cells showed a significant decrease in VCAM-1 expression (Figures 1, 3). This decrease was statistically significant (Figure 3) as compared to their respective *wt* controls ($p<0.005$). Furthermore, we observed no significant changes in the protein levels of laminin, FAK or N-cadherin with altered CRT expression (Figure 4).

Results from western blots were verified qualitatively by immunocytochemistry, which allows for the observation of relative protein levels, as well as their intracellular localization. Immunocytochemical staining of smooth muscle cells for vinculin displayed the same trend as in the western blots, with *SMCRT* cells having decreased levels of vinculin when compared to the control (Figures 5A and B). The decrease in vinculin was seen both at the focal adhesion sites (arrow, Figure 5A) and intracellularly. Western results for vinculin in *crt-/-* cells were also confirmed by immunocytochemistry

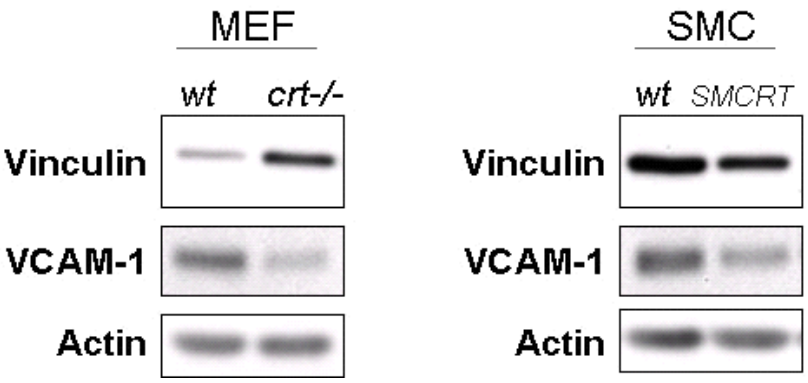

***Figure 1*. Effect of differential calreticulin expression on vinculin and VCAM-1 levels in mouse embryonic fibroblasts (MEF) and smooth muscle cells (SMC).** *crt-/-* mouse embryonic fibroblasts show a significant increase in vinculin levels as compared to *wt*, whereas VCAM-1 levels decrease in *crt-/-* cells. On the other hand, both vinculin and VCAM-1 levels are significantly decreased in *SMCRT* cells as compared to *wt*. Actin was used as a loading control.

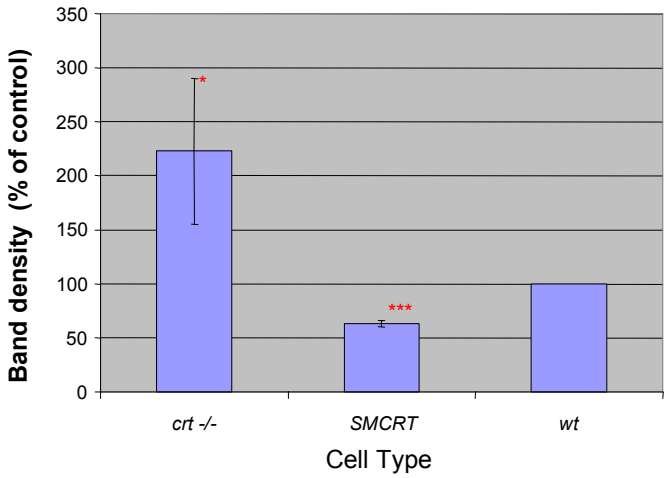

***Figure 2*. Vinculin levels vary with differential calreticulin expression.** Bands corresponding to vinculin and actin (loading control) were quantified using Bio Rad's QuantityOne software. The ratio of vinculin to actin band density was then calculated. Data are plotted as percentage of *crt-/-* or *SMCRT* cells to their respective *wt* cells. Values are mean ± SE of five experiments. *P<0.05; ***P<0.005, significantly different from control (*wt*).

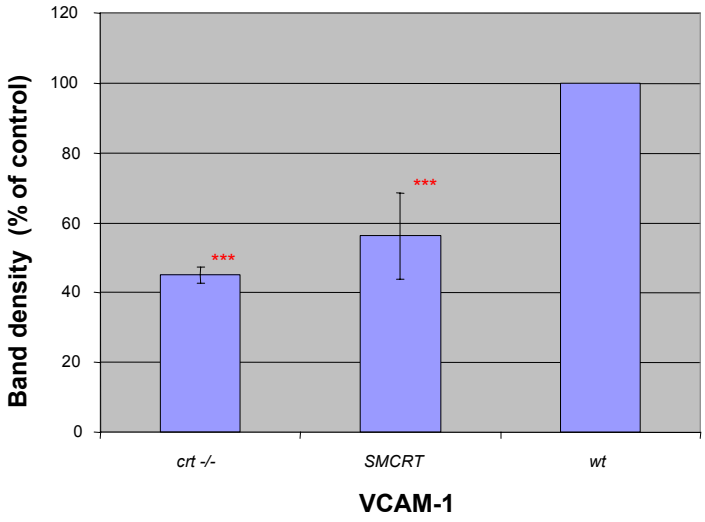

***Figure 3*. VCAM-1 levels decrease with both calreticulin overexpression and loss of expression.** Bands corresponding to VCAM-1 and actin (loading control) were quantified using Bio Rad's QuantityOne software. The ratio of VCAM-1 to actin band density was then calculated. Data are plotted as percentage of *crt-/-* or *SMCRT* cells to their respective *wt* cells. Values are mean ± SE of three experiments. ***P<0.005, significantly different from control (*wt*).

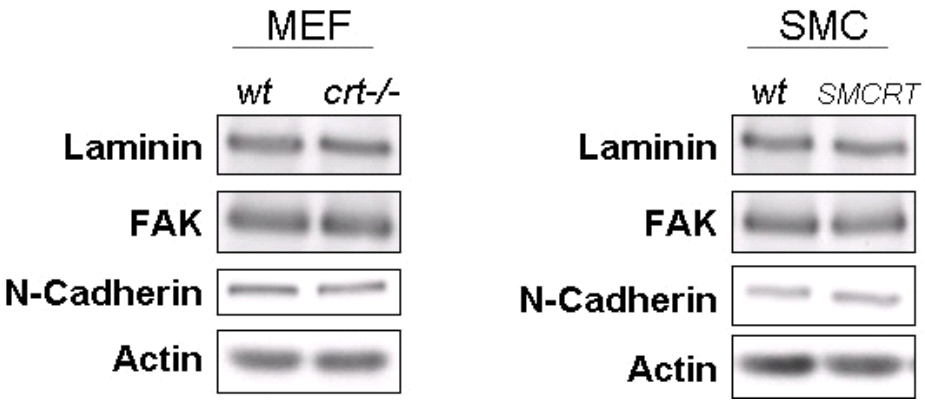

***Figure 4*. Varying calreticulin expression does not alter laminin, FAK, or N-cadherin levels.** No significant change was observed in either mouse embryonic fibroblasts or smooth muscle cells when compared to their respective *wt* cells. Actin was used as a loading control.

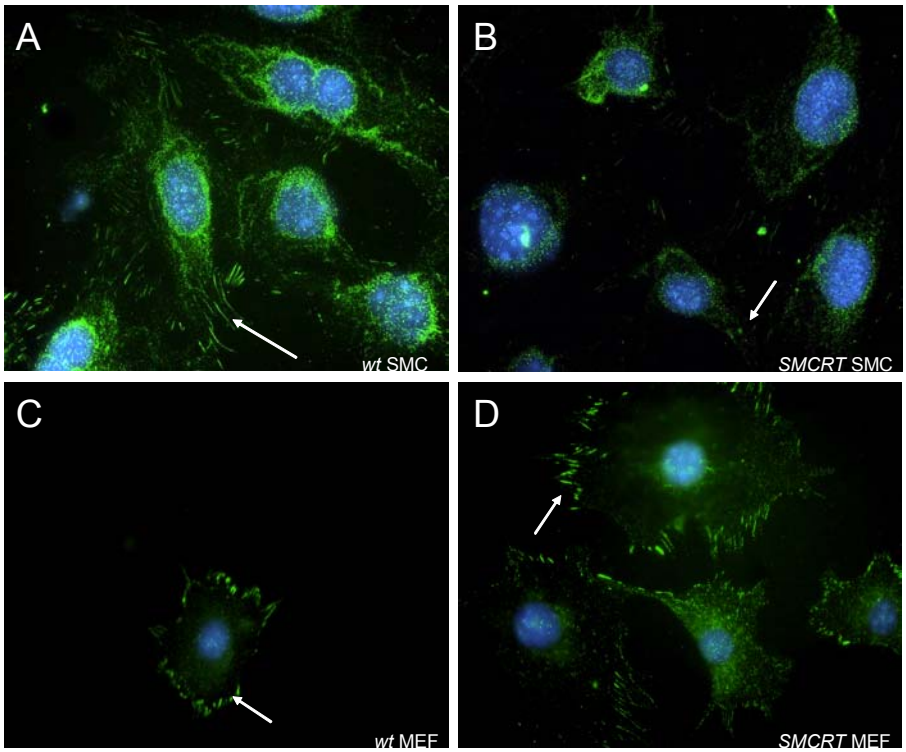

***Figure 5*. Immunocytochemical analysis demonstrates altered vinculin expression and localization with differential calreticulin expression.** All cells were labeled with anti-vinculin followed by a FITC conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). *wt* smooth muscle cells (A) show high levels of intracellular vinculin and numerous focal contacts. Vinculin expression in *SMCRT* smooth muscle cells overexpressing calreticulin (B) is nearly abolished, with very few focal contacts. *wt* mouse embryonic fibroblasts (C) show low levels of intracellular vinculin but do poses focal contacts. In *crt-/-* cells (D), there was increased intracellular vinculin levels and a higher number of focal contacts as compared to *wt* (C). Focal contacts are indicated by a white arrow.

(Figures 5C and D). *crt-/-* MEFs (Figure 5D) had an increase in number of focal contacts as well as higher levels of intracellular vinculin as compared to the *wt* (Figure 5C).

Immunocytochemical staining for VCAM-1 also supported the western data. The *SMCRT* cells (Figure 6B) appear to express less VCAM-1 than do *wt* cells (Figure 6A). Expression of VCAM-1 was localized to an area immediately surrounding the nucleus in *SMCRT* cells, most likely the ER. This observation needs to be confirmed via double labeling for VCAM-1 and the ER. In *crt-/-* MEF cells VCAM-1 was found localized to areas around the nucleus (Figure 6D), while VCAM-1 staining in the *wt* MEF cells was more dispersed, occurring throughout the cytosol in addition to areas surrounding the nuclei (Figure 6C). In the future, colocalization of VCAM-1 and an ER marker will be completed to determine the exact localization of VCAM-1 in MEF cells.

In order to verify that changes in CAMs detected in the cell lines were also apparent in the mouse tissue, immunohistochemical analysis was carried out. Frozen sections of descending aorta (7μm thick) from both *wt* and *SMCRT* mice were probed for VCAM-1 (Figure 7) and FAK (Figure 8). Expression of VCAM-1 is even and consistent along the luminal side of *wt* vessels, while non-uniform sporadic expression is observed in the *SMCRT* aortas. This difference is most easily observed in Figures 7C and 7D, where only VCAM-1 fluorescence is observed. Figures 7A and 7B show the same sections as 7C and 7D, but allow for a better appreciation of the overall histology of the sections. The autofluorescence of the elastin fibers (green) allows us to clearly see the bands of smooth muscle while the nuclei stain a bright blue. VCAM-1 in these images (7A and 7B) appears orange. These data support the results from both the western blots as well as the immunocytochemistry. Immunohistochemical staining could not be carried

out for vinculin, as this antibody is compatible only with acetone/methanol fixed samples, which is not appropriate for tissue fixation. Probing for FAK in both *wt* and *SMCRT* aorta sections confirmed that there is no significant change in FAK protein expression and localization between the *wt* and *SMCRT* (Figure 8).

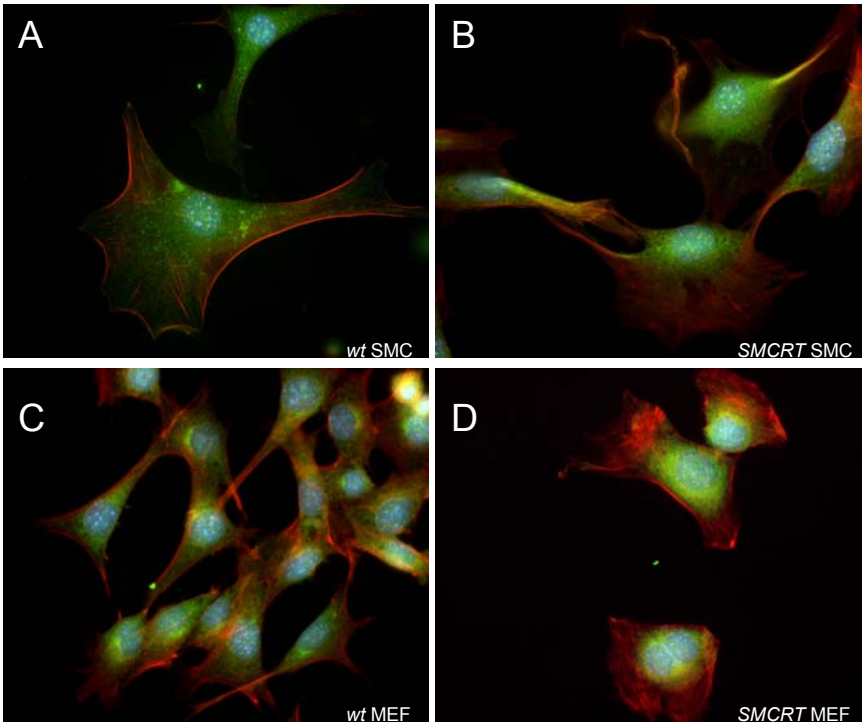

***Figure 6*. Calreticulin controls VCAM-1 levels.** Changes in levels of VCAM-1 expression are evident following double labeling with antibodies against VCAM-1 (green) and actin cytoskeleton (red). Cells were counterstained with the nuclear stain DAPI (blue). VCAM-1 in *wt* SMC (A) is diffusely localized throughout the cytoplasm. Expression is more tightly associated with the nucleus in *SMCRT* cells (B). *Wt* mouse embryonic fibroblasts (C) show a similar patern of staining to *wt* SMC cells (A), while in *crt-/-* cells VCAM-1 staining was condensed around the nuclei (D), similar to staining in SMCRT cells (B).

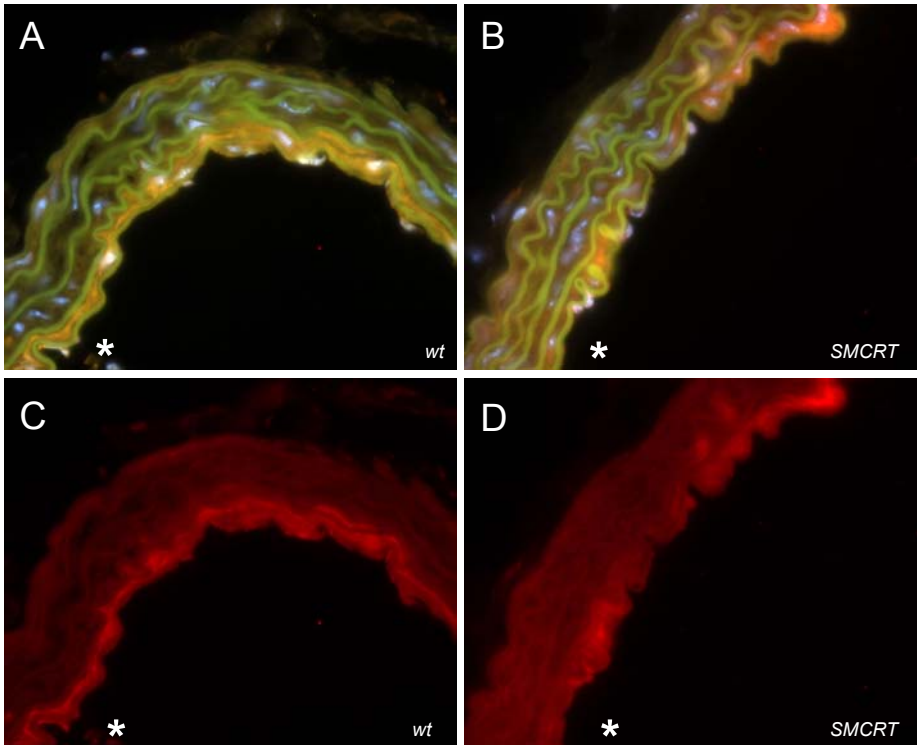

***Figure 7*. Immunohistochemical staining of VCAM-1 in the descending aorta.** Frozen section of *wt* (A, C) and *SMCRT* (B, D) descending aortas were labeled with an anti-VCAM-1 antibody (red). DAPI (blue) was used as a nuclear counter stain. VCAM-1 appears orange in both A and B due to the green autofluorescence of the elastin fibers within the bundles of smooth muscle. C and D allow for the viewing of VCAM-1 staining alone in *wt* and *SMCRT* descending aortas respectively. Specific staining is localized to the lumenal side of each vessel. Lumen is depicted by * in each section.

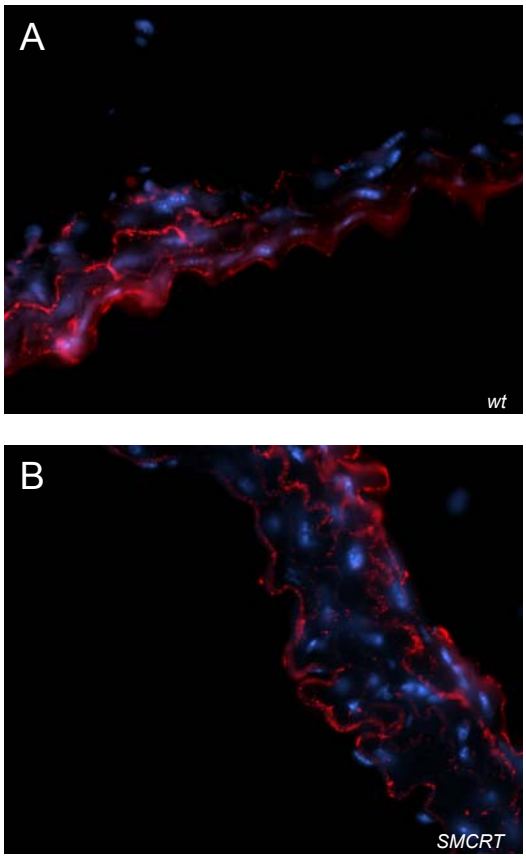

***Figure 8*. Immunohistochemical staining shows no difference in FAK expression in the descending aortas.** *Wt* (A) and *SMCRT* (B) descending aortas were probed with an anti-FAK antibody which was then visualized by a Texas Red-tagged secondary antibody. DAPI (blue) was used as a nuclear counter stain.

Discussion

CRT's role in controlling cell adhesion has previously been shown *ex vivo* (Coppolino *et al.,* 1997; Coppolino *et al.,* 1998; Fadel *et al.,* 1999; Fadel *et al.,* 2001; Michalak *et al.,* 1999; Opas *et al.,* 1996; Zhu *et al.,* 1997).This study provides evidence of the *in vivo* effect of CRT on the expression of cell adhesion molecules. Additionally, we have shown that CRT plays a specific role in regulating the cell adhesion of vascular smooth muscle cells. Both vinculin and VCAM-1 show significant changes in expression upon differential expression of CRT in vascular smooth muscle cells. Our results are however in direct contrast to several previously published studies (Fadel *et al.,* 1999; Fadel *et al.,* 2001; Opas *et al.,* 1996). It has previously been thought that overexpression of CRT results in elevated levels of vinculin and N-cadherin (Fadel *et al.,* 2001), and that elevated expression of CRT results in prominent focal contacts forming due to the elevated vinculin levels (Fadel *et al.,* 1999). These observations contradict our data, as we show that vinculin and focal contacts decrease in the CRT overexpressing (*SMCRT*) cells, and that CRT expression does not affect N-cadherin levels.

Vinculin

Previous studies have suggested that CRT may alter tyrosine phosphorylation within a cell (Fadel *et al.,* 1999; Fadel *et al.,* 2001; Opas *et al.,* 1996). Fadel *et al.* (1999) demonstrated that tyrosine phosphorylase activity decreases upon CRT overexpression. A recent study in our lab has shown that *crt-/-* cells have increased levels of protein tyrosine phosphorylation (Jalali and Mesaeli, unpublished observations). Altering the phosphorylation status of signaling proteins may affect signaling pathways that include vinculin. Protein phosporylation has been shown to regulate the stability and function of

cell adhesions (Fadel *et al.,* 2001). However, the same study also states that tyrosine phosphorylation effects are cell-type dependent. This cell-type specific effect may explain the discrepancy between their results and ours, as Fadel *et al.* (2001) were using L-fibroblasts whereas we studied smooth muscle cells. To date, no data is available on tyrosine phosphorylation in either *wt* or *SMCRT* smooth muscle cells.

It has also been suggested that CRT may regulate gene expression (Fadel *et al.,* 2001). This regulation is likely through CRT's role in controlling calcium homeostasis, as calcium is often involved in controlling transcription signaling pathways. This regulation of calcium may account for the 37% decrease in vinculin protein found in *SMCRT* cells. However, these results are still in conflict with the published data. CRT overexpression in L-fibroblasts significantly increased in vinculin mRNA levels (Fadel *et al.,* 2001). This disagreement may again be due to the use of different cell types. The other main difference is that in our study, CRT is expressed in the intact tissue while in Fadel *et al.* (2001), the L-fibroblast cell line is transfected with a plasmid containing CRT cDNA. Furthermore, signaling pathways leading to transcription may be different between our smooth muscle cells and MEFs and the L-fibroblasts used in Fadel *et al.'s* study (2001). CRT's role as a chaperone may influence the formation of focal contacts (Opas *et al.,* 1996). As we see in *SMCRT* cells, vinculin is localized to an area surrounding the nucleus, most likely the ER, and very few focal contacts. This may be a result of misfolding of vinculin due to the increased level of *CRT* which can cause an imbalance in the chaperone levels in the ER, causing the protein to accumulate within the ER. On the other hand, the decreased level of vinculin may be due to inhibition of vinculin transcription via *CRT* mediated altered calcium homeostasis.

Vinculin acts as a link between integrins and the ECM (Demali 2004; Moiseeva 2001), suggesting that vinculin plays a role in maintaining smooth muscle phenotype within the vasculature (Moiseeva 2001). In vinculin null mice, the turnover rate of focal adhesion is twice that of wild type mice, resulting in increased cell migration (Critchley 2004). *SMCRT* cells may have higher rates of migration than *wt* cells due to a decrease in vinculin levels, however, no data is currently available on *SMCRT* migration. This effect on migration could be examined in the future by means of a wound closure assay. Previously, increases in vinculin caused by elevated CRT levels resulted in increased cell spreading, while cells expressing low levels of CRT and vinculin did not spread as well (Fadel *et al.,* 1999; Fadel *et al.,* 2001). We have seen that SMCRT cells expressing high levels of CRT had low levels of vinculin and spread to a greater degree than did *wt* cells, which had high levels of vinculin and lower CRT expression (unpublished observation). This suggests that cell spreading may be directly affected by CRT via an effect on other cell adhesion molecules or increased cellular levels of calcium, and not via vinculin.

VCAM-1

VCAM-1 expression was significantly decreased upon both overexpression and deletion of CRT. To date, no other studies have proposed a link between CRT and VCAM-1. VCAM-1, expressed on both endothelial and smooth muscle cells of the vascular wall, regulates leukocyte migration from the blood into tissues via an interaction with α4β1-integrin molecules on leukocytes (Cook-Mills 2002; Moiseeva 2001). VCAM-1 is induced by cytokines, and there is evidence that the crosslinking of VCAM-1 stimulates a flux in intracellular calcium (Cook-Mills 2002). It has also been shown that VCAM-1 is important during development, as VCAM-1 knockout mice are embryonic

lethal (Cook-Mills 2002). The lack of consistent VCAM-1 expression in the aorta of the *SMCRT* mouse suggests there is a reduced ability to take up leukocytes into the vessel, and thus the mice are less able to combat infecion. This may explain the high incidence of infection following skin hemangioma seen in *SMCRT* mice. The lower levels of VCAM-1 observed in *SMCRT* and *crt-/-* cells suggest that CRT protein is the chaperone for VCAM-1 protein. On the other hand, the decreased level of VCAM-1 may be due to its rapid degradation. Indeed, both *crt-/-* and *SMCRT* cells show increased activation of the proteasome degradation pathways (Uvarov and Mesaeli, unpublished observation). These are interesting possibilities which will be investigated in the near future.

N-cadherin, FAK, and laminin

N-cadherin mediated cell adhesion occurs primarily in a homophilic and homotypic manner, with binding specifically between N-cadherin molecules on opposing cells (Radice *et al.,* 1997). N-cadherin appears to play a critical role during early mouse heart development (Radice *et al.,* 1997). We have shown that levels of N-cadherin do not change despite changes in the expression of CRT. This result disagrees with the literature, which shows an increase in N-cadherin levels upon overexpression of CRT (Fadel *et al.,* 2001). This difference, as with the differences in vinculin expression between our work and the previous study, may be due to differences in cell type. It also could be due to the experimental method (cell transfection as opposed to transgenic mice). Our observations that levels of FAK do not change upon differential expression of CRT are supported by several studies ((Fadel *et al.,* 1999; Fadel *et al.,* 2001; Opas *et al.,* 1996).

Laminin levels are not altered by changes in CRT levels. To the best of our knowledge, no other study has examined the effects of differential CRT expression on intracellular laminin levels. It has been demonstrated however, that CRT serves as a calcium dependent chaperone of laminin (McDonnell *et al.,* 1996). The study suggests that individual laminin subunits are bound by CRT and that they are complexed while bound to CRT (McDonnell *et al.,* 1996). No evidence of this is presented, nor is it shown that the presence of CRT is obligatory for proper laminin formation. It is possible then that CRT is able to transiently bind laminin, without playing a role in protein folding, which is suggested by lack of change in laminin protein levels upon variable CRT expression. Alternately, in *crt-/-* cells calnexin (another ER chaperone which assists CRT in lectin-like chaperone activity) is increased significantly (Knee *et al.,* 2003), which might assist in laminin folding in the absence of CRT.

Hemangioendothelioma

Our data show support for the hypothesis that change in levels of cell adhesion molecules within the vascular wall of *SMCRT* mice contributes to the development of hemangioendothelioma. Both vinculin and VCAM-1 have previously been implicated in tumour development (Ding *et al.,* 2003; Rodriguez Fernandez *et al.,* 1992). Vinculin null cells are resistant to apoptosis (Subauste *et al.,* 2004), and vinculin acts as a tumour supressor (Rodriguez Fernandez *et al.,* 1992). Interestingly, we showed decreased vinculin expression in smooth muscle cells of the *SMCRT* mouse, which could contribute to the development of hemangioendothelioma in *SMCRT* mice. It has also been noted that vinculin deficient cells are highly metastatic and motile (Rodriguez Fernandez *et al.,* 1993; Subauste *et al.,* 2004). Increased cell plasticity and motility in cells of the vascular

wall of *SMCRT* mice may partially explain the growth of hemangioendotheliomas, at the base of the aorta and many other organs, containing endothelial cells and thin-walled vessels filled with sheets of endothelial cells. Cell-cell and cell-ECM interaction via integrins have been implicated in tumour progression through signaling pathways (Guo *et al.,* 2004). Vinculin can function as a link between the integrin dimer and the cytoskeleton, thus it is possible that changes in vinculin levels could be responsible for altering the signaling pathways. Additionally, the ECM is implicated in maintaining the vascular smooth muscle phenotype (Moiseeva 2001). With reduced ECM-integrin focal contacts as a result of low vinculin levels, *SMCRT* smooth muscle cells would not be in strong contact with the ECM. This may result in altering smooth muscle cell and endothelial cell phenotypes in the arteries of the *SMCRT* mice and aiding in the development of hemangioendothelioma.

VCAM-1 overexpression has previously been associated with oncogenesis, tumour angiogenesis, and metastasis (Ding *et al.,* 2003). The smooth muscle of *SMCRT* mice had decreased VCAM-1 levels (Figure 1) which may affect the ability of the tumour-fighting leukocytes to penetrate into the vascular wall and prevent the hemangioendothelioma. Controlling VCAM-1 levels may be essential for maintaining normal immunological responses and preventing development of hemangioendothelioma.

Future directions

This study has shown that altering CRT levels alters CAM levels of the vascular smooth muscle cells of *SMCRT* mice. The mechanisms that result in these changes however are not understood. Future studies will address how CRT is effecting these changes. Such studies may include determining the phosphorylation levels of signaling

molecules involved in cell adhesion (e.g. FAK), changes in rate of *SMCRT* cell adhesion and cell migration. In addition, studies focused on determining the precise effects of vinculin and VCAM-1 in progression of these tumours are needed. A better understanding of this mouse model will allow for the future development of new therapies to better treat and control hemangioendotheliomas.

Conclusions

1. Calreticulin significantly decreases the levels of both vinculin and VCAM-1 in the vascular wall of transgenic mice overexpressing calreticulin in vascular smooth muscle cells (SMCRT). The regulation of these cell adhesion molecules may be effected by calreticulin in several ways:
    a. Calreticulin may alter the expression at the mRNA level of these cell adhesion molecules.
    b. Calreticulin may alter the folding and maturation of newly synthesized cell adhesion proteins.
    c. Calreticulin may affect the maturation of cell adhesion molecules by altering tyrosine phosphorylation.
2. Overexpression of calreticulin in vascular smooth muscle did not elicit any changes in the levels of laminin, FAK or N-cadherin proteins. Furthermore, gene targeted deletion also did not alter the expression level of these proteins, indicating that calreticulin's effect on cell adhesion is not mediated via laminin, FAK, or N-cadherin..

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