

Tissue repair cells for the treatment of cardiovascular diseases

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One approach to tissue regeneration involves expansion of a patient's own stem and progenitor cells outside of the body to generate a cell product with significantly increased numbers of highly functional early-stage cells. These autologous cell products can then be used for the repair or regeneration of multiple human tissues. Aastrom has developed a proprietary Tissue Repair Cell (TRCs) technology. TRCs are derived from a small amount of bone marrow, taken from a patient and expanded in culture to generate a unique cell mixture containing high doses of stem and progenitor cells. A proprietary cell manufacturing platform has been developed for TRCs which is closed, automated and GMP compliant. TRC-based products have been used to treat over 240 patients, and are currently in clinical trials for bone regeneration (osteonecrosis of the femoral head and long bone fractures) and vascular regeneration (critical limb ischemia) applications. Due to the high number of stem and progenitor cells contained in a small volume, the product seems particularly suitable for direct injection into remodeled myocardium. The first TRC applications in this setting are expected to start soon.

Key words: Adult stem cells, tissue regeneration, vascular, cardiac, bone marrow

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Introduction

Aastrom is a regenerative medicine company developing autologous cell products for the repair or regeneration of multiple human tissues, based on its proprietary Tissue Repair Cell (TRCs) technology (Figure 1). Aastrom's TRC-based products are derived from a small amount of bone marrow, taken from the patient, which is expanded in culture to generate a unique cell mixture containing high doses of stem and progenitor cells. TRC-based products have been used to treat over 240 patients, and are currently in clinical trials for bone regeneration (osteonecrosis of the femoral head, long bone fractures and spine fusion) and vascular regeneration (critical limb ischemia) applications. Aastrom has reported positive interim clinical trial results for

TRCs suggesting both the clinical safety and the ability of TRCs to promote healing. The Company is also developing clinical programs for TRC-based therapies to address cardiac and neural regeneration indications that should begin treating patients over the next 12 months. TRCs have recently received Orphan Drug Designation from the FDA for use in the treatment of osteonecrosis of the femoral head and the treatment of dilated cardiomyopathy, a severe chronic disease of the heart.

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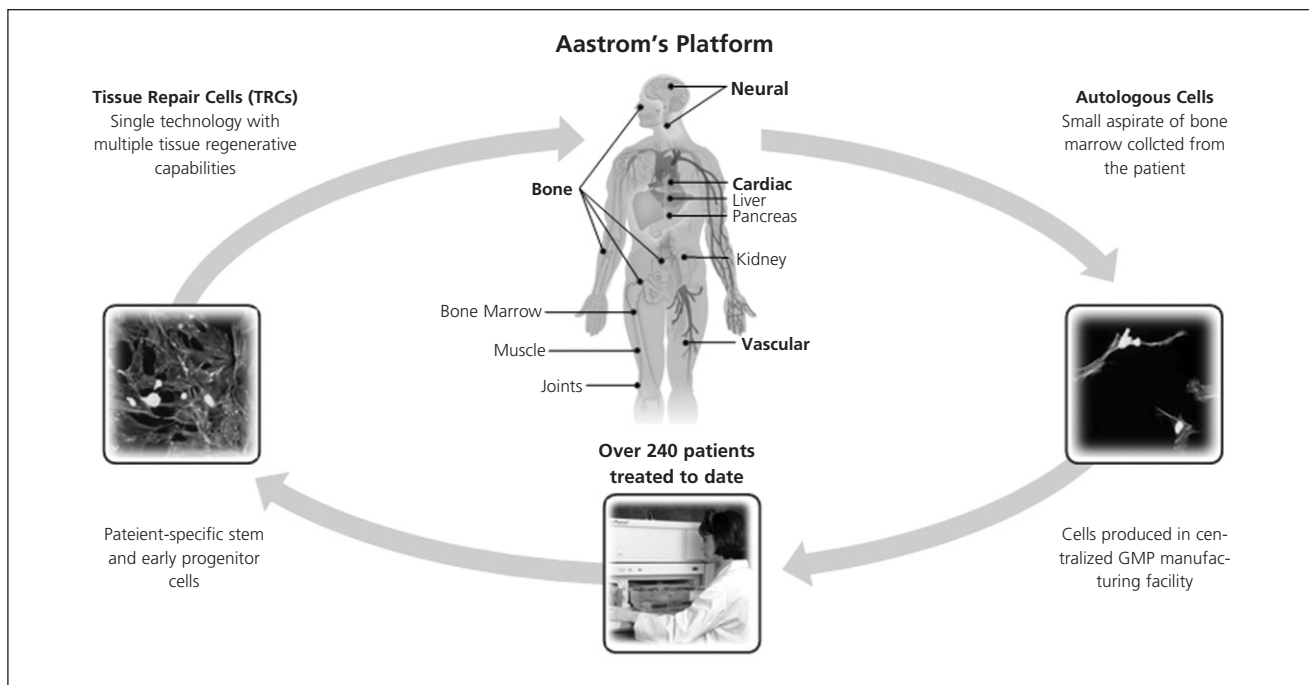


Figure 1

Schematic of Aastrom's Approach to tissue engineering

Adult tissues contain limited numbers of stem and progenitor cells for endogenous regeneration in the event of injury. These cells are normally found in the bone marrow, blood and many organs and tissues, and are sufficient to support normal tissue turn-over and typical wound healing processes but are insufficient to regenerate many severely damaged or diseased tissues. The inability to fully regenerate is likely an evolutionary limitation related to the total number of stem cells present in the body as well as the ability of those cells to migrate to the site of injury in significant numbers. Aastrom's approach to tissue regeneration involves expansion of a patient's own stem and progenitor cells outside of the body to generate a cell product with significantly increased numbers of highly functional early-stage cells. These cells can then be delivered directly to the tissue of interest either alone, or in combination with biomaterials, to promote regeneration.

Tissue Repair Cells (TRCs)

Tissue Repair Cells (TRCs) are a mixture of stem and progenitor cells derived from a patient's own

bone marrow. TRCs are generated from bone marrow mononuclear cells (BM MNC) in a single-step 12 day culture process with a ramped medium perfusion schedule. The ramped single-pass perfusion (SPP) schedule for the culture medium results in significant expansion of primary early-stage human cells possessing enhanced functionality (see below). Unlike other cell expansion techniques, this process results in a unique mixture of highly functional primary human cells.

Single-Pass Perfusion (SPP) Technology

In SPP, culture medium is continuously replaced by fresh medium at a slow, controlled rate without disturbance or removal of cells, enabling optimal exchange of nutrients and metabolic by-products and maintenance of the bone marrow-like microenvironment. Figure 2 shows the importance of perfusion rate for the optimal production of primitive hematopoietic stem cells (LTC-IC) in long-term bone marrow cultures.¹

The use of this method of culture, along with uniform oxygenation in a batch or continuous perfusion mode, results in high density cultures with superior biological activity in a variety of human primary cell culture systems:

- Medium perfusion is critical for the productivity and longevity of bone marrow (BM) cultures,² and enhanced metabolic activity and growth factor production rates of BM stromal cells.³

- Monocyte-derived dendritic cells have enhanced antigen presentation and maturation with increased medium perfusion.⁴

- Continuous perfusion in human T-lymphocyte cultures yield very high density cultures with superior replicative potential and enhanced biological function.⁵

TRC Characterization

TRCs contain a variety of cell types from hematopoietic, mesenchymal, and endothelial lineages. The TRC culture process results in a significant increase in the frequency of key stem and progenitor cell lineages with an overall decrease in total nucleated cell numbers.

Numerous in vitro methods including flow cytometry, cell counting, cell viability measure-

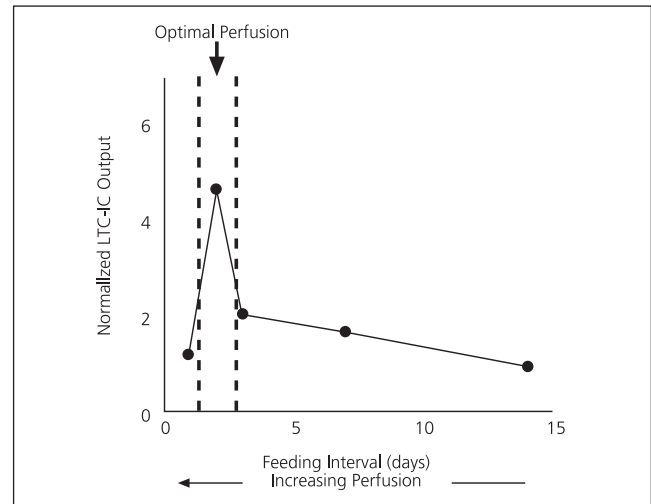


Figure 2

Effect of medium perfusion schedule on stem cell production. BM MNC were cultured for 14 days with different feeding schedules. Output of LTC-IC was measured from each culture and normalized output is shown.

ments, proliferation, colony forming ability, and differentiation potential are used to characterize the TRC product. Preclinical animal models have also been employed to establish the role of TRCs in bone and vascular tissue regeneration.

The typical phenotype, measured by flow cytometry for cell surface markers, of starting BM MNC and the TRC product is shown in Table 1.

Table 1

Phenotype of TRCs generated under SPP conditions

Lineage	Marker	BM MNC Input		TRC Output		
		%	Total (in millions)	%	Total (in millions)	Fold Expansion
	Total Cells		277		130	0.5
	Viability	96		87		
M	CD105/166	0.03	0.1	12	16	373
H	CD14auto+	0.2	0.5	26	36	81
M	CD90	0.4	0.9	22	28	39
H (E)	CXCR4/VEGFR1	0.7	1.9	12	9.9	21
E	CD144/146	0.5	1.3	2.7	3.2	6.3
E	VEGFR1	7.6	22	26	38	2.3
E	VEGFR2	12	37	25	37	1.3
H	CD14auto-	11	31	14	17	0.9
H	CD11b	59	162	64	83	0.5
H	CD45	97	269	80	104	0.4
H	CD3	24	67	8.6	11	0.2
H/E	CD34	4.4	12	1.1	1.5	0.1

M: mesenchymal lineage, **H:** hematopoietic lineage, **E:** endothelial lineage. Results are the average of 4 clinical-scale experiments.

Briefly:

- Expansion under SPP conditions results in a high viability cell product.
- Mesenchymal components (CD105+/166+ and CD90+), along with macrophage CD14^{auto}+ populations are significantly expanded during SPP culture.
- A dual positive CXCR4 and VEGFR1 positive cell, believed to be critical for neovascularization, is increased in TRCs.⁶
- Cells expressing endothelial cell markers (VEGFR1+, VEGFR2+, and CD144+/146+) are increased up to 6-fold in TRCs.
- Many hematopoietic components are reduced although still present in TRCs, including T-cells (CD3+), B-cells (CD19+, not shown), mature myeloid cells (CD11b+, CD45+), and primitive hematopoietic cells (CD34+).

The ability of cells within TRCs to form clonogenic colonies was also measured. Both hematopoietic (CFU-GM) and mesenchymal (CFU-F) colonies were monitored (Table 2), and, while CFU-F were increased 280-fold, CFU-GM were slightly decreased by culturing.

In addition to the unique mixture of enhanced stem and progenitor cells, a key feature of the TRC expansion process is a significant reduction in product variability compared to starting bone marrow isolated directly from the patient.

Within a single donor, the TRC expansion process is reproducible and does not add to the inherent donor variability seen in bone marrow aspirates (approximately 33% across 26 donors).

For certain cell types, including the mesenchymal CD90+ cells, not only are the numbers of these cells substantially increased, but the donor variability is reduced from 85% in the starting bone marrow population to 40% in the TRC product, providing a more standardized product for all patients (Figure 3).

Vascular Regeneration

Diseases and traumatic injuries that lead to the destruction of vascular tissue cause numerous major limb amputations every year. Therapeutic options are limited and largely ineffective for the most severely affected patients. TRC-based products are currently developed with the goal of repairing

Table 2
Average colony forming ability of TRCs

	BM MNC Input(E-06)	TRC Output (E-06)	Fold Exp
CFU-GM	1.7	1.1 ± 0.2	0.7 ± 0.1
CFU-F	0.03	6.7 ± 1.3	280 ± 67

Results are the average ± SEM from 8 clinical-scale experiments.

and regenerating the ischemic tissues of these patients by improving the blood flow in the affected areas.

Bone marrow-derived cells are known to enhance neovascularization in models of myocardial and peripheral ischemia. Cultured cells can incorporate into sites of neovascularization in ischemic tissues, preserving left ventricular function in the ischemic heart⁶ and enhancing blood flow in ischemic limbs.^{7,8} There is enormous potential for a clinically-suitable cell therapeutic for use in vascular applications to treat ischemic or damaged tissue, as well as in tissue engineering of replacement organs.

In vitro vascular potential of TRCs

Preliminary studies have examined the potential of TRCs to promote vessel development, either

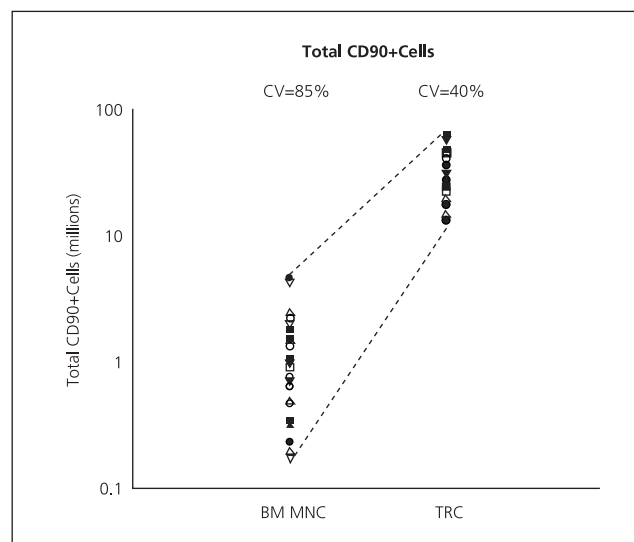


Figure 3
Total numbers of CD90+ cells were measured in the starting BM MNC and TRC product from 26 donors. Results show less variability and higher levels of CD90+ cells in TRCs compared to BM MNC.

through the production of angiogenic factors, or through the production of cells which have the capacity to associate with or incorporate into developing vessels. Results from these studies have shown:

- Angiogenic factors including, vascular endothelial growth factors (VEGFs A, B, and C), Leptin, IL-6, Neuropilin 1 (NRP1), and G-CSF are expressed in TRCs as measured using targeted microarrays. These transcripts are present at higher levels than in BM MNC.

- Some angiogenesis inhibitors, including thrombospondin-1 (TSP-1) are also upregulated. It is known that a balance between positive and negative regulators is critical for the generation of functional stable vessels.

- TRCs can form tube-like structures in matrigel in the presence of endothelial growth factors (Figure 4)

- Cells within TRCs can generate endothelial progenitor cell colonies (Figure 4). Colonies are identified by round cells in the center with spindle-shaped cells at the periphery.

In vivo vascular potential of TRCs in ischemia models

Model of acute ischemic myocardial infarction

In collaboration with Boston Scientific and Harvard Cardiovascular Research Center, a myocardial infarction (MI) was induced in male athymic

Table 3
Effect of TRCs on cardiac function

Readout	TRCs	Sham
end-systolic elastance (mmHg/ μ L)	0.45 \pm 0.05	0.20 \pm 0.03
maximal elastance(mmHg/ μ L)	1.15 \pm 0.2	0.42 \pm 0.08
preload-recruitable stroke work (mmHg)	92.7 \pm 11.3	48.3 \pm 15.1

nude rats (n=27) by ligation of the left anterior descending coronary artery. One week after MI, 2.5x10⁶ (low-dose) or 5x10⁶ TRCs (high-dose) were injected into the infarct tissue, while a third group of rats were given injections of vehicle in which cells were absent (sham control). Rats were studied 5 weeks after cell therapy.

Results showed that implantation of high dose TRCs resulted in:

- Anterior wall thickening was increased by 170 \pm 60% compared to sham.

- Significant improvement (p<0.05) of load insensitive measures of cardiac contractility, integrated pump function and arterial elastance high-dose vs. sham (Table 3).

In all, these data suggest that implanted TRCs formed viable grafts in myocardial infarcts. When sufficient cells were provided (high-dose group) regional and global contractile function were enhanced and ventricular dilation attenuated.

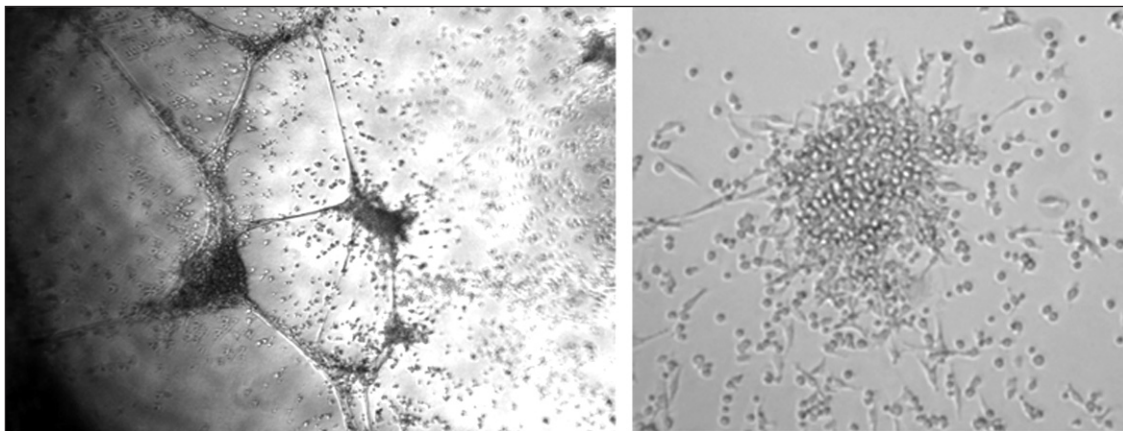


Figure 4

TRCs can form tube-like structures and give rise to endothelial progenitor colonies in vitro. TRCs were plated in matrigel for up to 24 hours and assessed for tube formation (left panel). For endothelial progenitor colony assays (right panel), the CFU-Hill assay was followed (Stem Cell Technologies, Vancouver, BC).

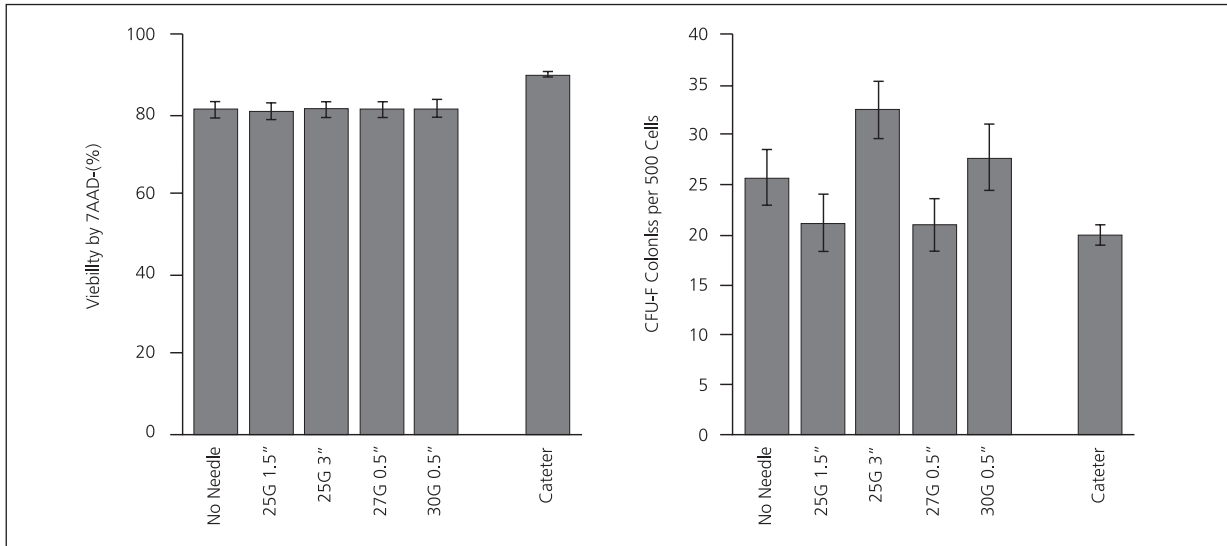


Figure 5

TRCs maintain viability and colony forming potential after passing through different size needles. No significant differences were observed between No Needle (control) and test groups. The catheter was 67" long with a 28G needle.

Cell delivery for vascular applications

To be effective, cells must be administered while maintaining their viability and functionality. For vascular applications, cells will be injected through small gauge needles directly into muscle tissue. We have completed several studies showing that TRCs survive passing through needles and remain functional (Figure 5).

Clinical vascular regeneration experience

Based on Aastrom’s observations that TRCs have the ability to form small blood vessels, and third party trials involving the use of bone marrow cells for peripheral vascular disease, a trial to evaluate the safety and efficacy of TRCs in the treatment of diabetics with open wounds and critical limb ischemia was initiated.



Figure 6

Toe of 69 year old male patient treated with TRCs. Before treatment (left) a non-healing wound was observed. 44 weeks after treatment (right) complete healing was observed. The patient suffered from numerous co-morbidities including coronary heart disease, chronic heart failure, hypertension and hyperlipidemia.

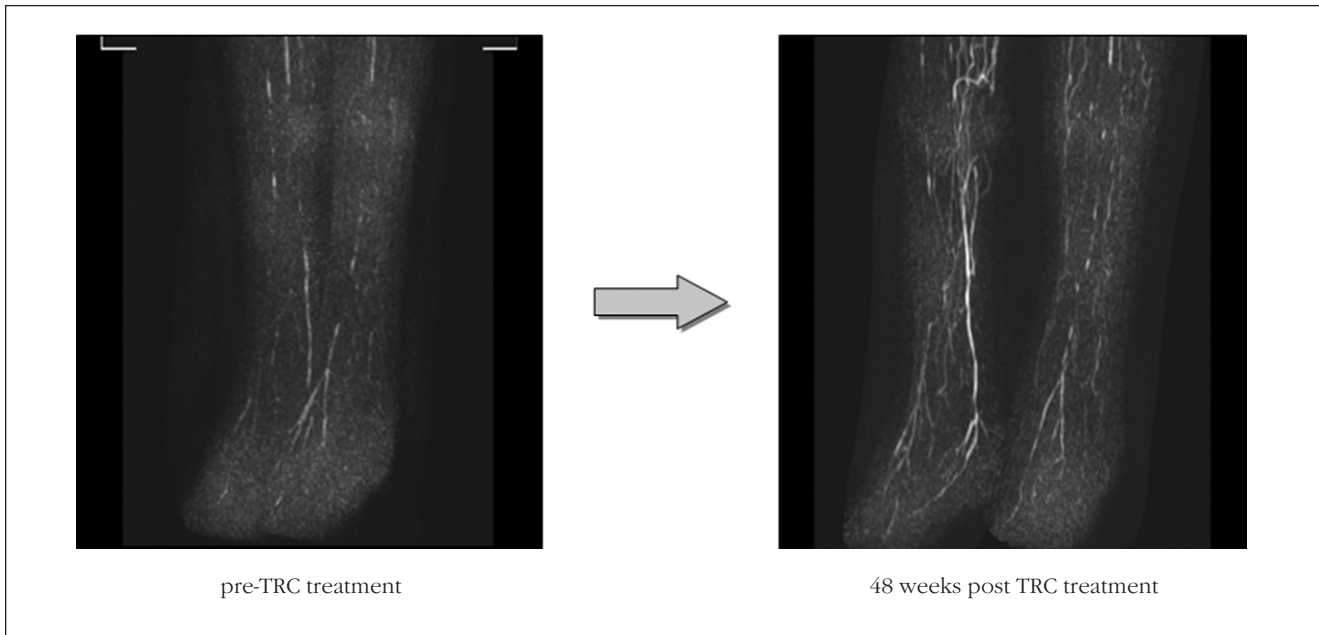


Figure 7

MR-angiography of limbs of 69 year old male patient treated with TRCs. This patient received TRC injections in the right limb. Before treatment (left panel) very little collateralization is observed. 48 weeks after treatment (right panel) significantly more collaterals can be observed in the treated limb. The patient suffered from numerous co-morbidities including coronary heart disease, hypertension and hyperlipidemia.

Aastrom entered into a clinical trial agreement with the Heart & Diabetes Center located in Bad Oeynhausen, Germany, to conduct a pilot trial to evaluate the safety and potential efficacy of TRCs to improve peripheral circulation in diabetic patients with open wounds and critical limb ischemia. Patients were enrolled if they had an open wound that had not healed and showed no tendency to heal for at least 6 weeks prior to enrollment. Patient enrollment of up to 30 patients is ongoing.

Interim data including the first two patients treated with TRCs was presented at the 19th World Diabetes Congress in Cape Town South Africa in December 2006.⁹ Briefly, the investigators reported that the patients treated with TRCs healed their non-healing open wounds in 48 and 44 weeks respectively (Figure 6) and showed improvement in collateral vessel formation (Figure 7). The current standard of care arm of this trial showed no healing of open wounds.

Aastrom is in the process of preparing a phase IIb clinical trial protocol in the U.S. to treat patients

suffering from critical limb ischemia with the goal of reducing of the incidence of major amputations.

Future TRC Applications

The nature of TRCs makes them ideally suited for a broad list of potential applications. As a high-dose mixed autologous bone marrow-derived cell population it is expected that the TRCs will 1. have an excellent safety profile since these are the patient's own cells, 2. have the ability to work in any indication where bone marrow-derived cells have suggested a clinical effect since all of the components of bone marrow are still present in TRCs, and 3. have greater clinical efficacy due to the high doses of stem cells that TRCs bring to the tissue in need of regeneration.

Potential indications include a number of trauma-related applications that could benefit patients:

- Post-traumatic limb salvage where bone, vascular, connective and nervous tissues may all be effected.
- Severe orthopedic trauma effecting long bones, the spine or craniomaxillofacial bone.



Figure 8

Pictured are a single use cell cassette with medium reservoir (left) and the full cell production system being loaded with a batch of cells (right).

- Degenerative bone disease such as osteonecrosis which is a common post-traumatic condition

- Non-healing wounds and ischemic tissue regeneration related to vascular damage.
- Articular cartilage regeneration
- Skeletal muscle regeneration
- Neural regeneration including peripheral nerves and spinal cord injuries.

Other potential applications may address serious chronic conditions:

- Cardiac regeneration to limit the effects of myocardial infarction or reverse chronic heart disease
- Vascular regeneration to reverse critical limb ischemia associated with diabetes
- Liver regeneration
- Kidney regeneration
- Pancreas regeneration

Manufacturing Platform For Production of Tissue Repair Cells

The practical delivery of an ex vivo cell therapy requires a comprehensive cGMP-compliant cell manufacturing process that integrates 1. the biolog-

ical process that drives production of functional cells in sufficient numbers, 2. the culture device capable of supporting the biological process, and 3. an automated support system which provides reliability and reproducibility of the process in a closed system environment.

Astrom is in a unique position in the field of cell therapy, having developed a closed, automated GMP compliant system for the production of stem and progenitor cells.¹⁰ Astrom's cell manufacturing system embodies a modular, closed-system process comprised of a pre-sterilized, single-use disposable cell cassette operated by automated instruments (Figure 8). The bioreactor within the cell cassette implements single-pass perfusion (SPP) for optimal cell growth. The cell cassette provides a functionally closed, sterile environment in which cell production occurs. The fluid pathway in the cell cassette includes the cell growth chamber (bioreactor), a medium supply container, a mechanism for medium delivery, a waste medium collection container, and a container for the collection of harvested cells. All components are interconnected with sterile barrier elements throughout to protect the culture from contamination during use.

Automation of the cell culture process has resulted in reliable and reproducible cell production, limiting the amount of cell manipulation and also limit-

ing donor variability. In total Aastrom's manufacturing system, has supported more than 625 ex-vivo cell production lots for over 240 patients.^{11,12}

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