Published in final edited form as:

Proteomics. 2012 February ; 12(4-5): 722–735. doi:10.1002/pmic.201100346.

Secreted proteins as a fundamental source for biomarker discovery

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Abstract

The proteins secreted by various cells (the secretomes) are a potential rich source of biomarkers since they reflect various states of the cells at real time and at given conditions. To have accessible, sufficient and reliable protein markers is desirable since they mark various stages of disease development and their presence/absence can be used for diagnosis, prognosis, risk stratification and therapeutic monitoring. As direct analysis of blood/plasma, a common and noninvasive patient screening method, can be difficult for candidate protein biomarker identification, the alternative/complementary approaches are required, one of them is the analysis of secretomes in cell conditioned media *in vitro*. Since the proteins secreted by cells as a response to various stimuli are most likely secreted into blood/plasma, the identification and preselection of candidate protein biomarkers from cell secretomes with subsequent validation of their presence at higher levels in serum/plasma is a promising approach. In this review, we discuss the proteins secreted by three progenitor cell types (smooth muscle, endothelial and cardiac progenitor cells) and two adult cell types (neonatal rat ventricular myocytes and smooth muscle cells) which can be relevant to cardiovascular research and which have been recently published in the literature. We found, at least for secretome studies included in this review, that secretomes of progenitor and adult cells overlap by 48% but the secretomes are very distinct among progenitor cell themselves as well as between adult cells. In addition, we compared secreted proteins to protein identifications listed in the Human Plasma PeptideAtlas and in two reports with cardiovascular-related proteins and we performed the extensive literature search to find if any of these secreted proteins were identified in a biomarker study. As expected, many proteins have been identified as biomarkers in cancer but 18 proteins (out of 62) have been tested as biomarkers in cardiovascular diseases as well.

Keywords

conditioned media; secreted proteins; proteomics; biomarker discovery

1 Introduction

The proteome is defined as the “complete” set of proteins expressed by cell, tissue or organism. It can also be used to refer to specific sub-cellular proteins (the subproteomes) such as proteins of the same organelle, cellular location or specific post-translational

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Conflict of interest statements

None.

modification (PTM). The set of proteins secreted by the cell, termed the secretome, is one of the cellular subproteomes but this term can be broader as it can often include the proteins of extracellular matrix (ECM) as well as additional proteins shed from the cell surface [1]. Similarly to the proteome, the secretome is highly dynamic and it changes in protein expression during organ development, maturation and during various pathologies and environmental stimuli. It also can reflect the state of the cells at various stages of disease progression. The secreted proteins have the important roles in cell-cell signaling, communication, growth and as they reflect the various stages of pathological conditions [2], they are incredible source for biomarker search and a novel discovery. In addition to common sources of biomarkers (blood/plasma, other body fluids and tissues), various novel approaches have been used to target biomarkers, such as analysis of proximal fluids [3–6], exosomes [7], membrane vesicles [7] and conditioned media with secreted proteins from cultured and primary cells *in vitro* [8]. Many of the potential protein biomarkers identified from these sources might be found circulating in the blood, which is a preferable way of patient clinical screening [9]. And so, the identifications of protein biomarker candidates in discovery stage are verified and their levels compared to corresponding proteins in blood/plasma during subsequent verification and validation steps.

Most often, the proteins secreted by cells of interest into conditioned media *in vitro* are studied to better understand the mechanisms *in vivo*. Although *in vitro* conditions can only partly mimic *in vivo* environment, it is still a good option to map the quality and quantity of the secretome and provide insight into possible biological pathways involved. As well, various assays and disease models *in vitro* have been developed to simulate the pathological conditions *in vivo*. For example, induced hypoxia or hyperoxia and oxidative stress or cyclical stretch assays can simulate the cardiac function impairment. As well, the *in vitro* assays were developed to monitor the effect of various secreted factors or whole secretomes on the other cells e.g. proliferation, differentiation, and cellular protection against injury. Nevertheless, one of the challenges related to secretome analysis *in vitro* is to distinguish the secreted proteins from intracellular proteins arising from the cell death and from proteolysis induced during the cell culture handling. As well, the conditioned medium can contain proteins shed from the membrane and secretory vesicle proteins (e.g. from exosomes and microsomal vesicles) which then can constitute the fraction of the secretome [7].

With maturation of proteomics field [10] and progress in methodology and instrumentation, the present techniques have been improved and novel methods developed with efforts to mine the secreted proteins present very often in low concentrations. In addition, novel methods for *in vivo* secretome sampling have been recently tested, e.g. based on peptide nanofiber hydrogel as an injectable delivery agent. As well, bioinformatics and the ability to handle large quantity of data obtained by mass spectrometry (MS), linking the data to known databases, the secreted/extracellular protein- and biomarker-based prediction models, and the incorporation the biological pathways to put the protein identifications into the broader context with known or predicted mechanisms *in vivo*, contribute to the efforts for secreted proteins identification and biomarker discovery.

2 Secretomes of the cells related to cardiovascular research

The basic steps involved in secretome analysis strategy are depicted in Figure 1. Figure 1 schematically shows the approach for analysis and identification of proteins secreted into conditioned medium of various cells but it can be modified and applied based on specific needs and goals. The *in vitro* experiment conditions involve the cell culturing (10–20% of serum; FBS, FCS), cell conditioning (serum-free [11–12] or low serum medium (1–2%) [13]; mostly for period of 12–48 hours [13–15]), conditioned medium collection and concentration, medium fractionation by various protein separation methods and the

identification of secreted proteins in the conditioned medium by MS. The high abundant serum proteins in conditioned medium can be removed (e.g. antibody-affinity chromatography approach) and the protein(s) of interest enriched (e.g. antibody or selective PTM enrichment approach). The technical issues and challenges related to identification of secreted proteins in cell conditioned media including separation methods for media pre-fractionation and MS techniques [16] for protein identification with description of e.g. stable isotope labeling by amino acids in cell culture (SILAC) [17] and label-free quantitative MS-based techniques such as spectral counting and multiple reaction monitoring (MRM) [18], have been described in details previously [19] together with the follow-up on bioinformatic approaches for further protein characterization and on relevant protein biological pathway analysis softwares.

Many studies which centered on either one or more targeted or known proteins have been reported and the effects of selected proteins/factors on the cell performance and function (both promotive and inhibitive) have been demonstrated and verified by various approaches (e.g. Western blotting, ELISA, (micro)arrays and gene expression profiling). In this review, we will rather concentrate on studies of cell conditioned media secretomes relevant to cardiovascular research in which the larger sets of proteins were identified by using MS techniques – studies which provide a broader view on the secretome. As well, for the purposes of this review, the authors excluded consideration of exosomes and microsomal vesicles (secreted proteins containing particles).

2.1 Smooth muscle cells

The global map of the proteome and secretome of primary culture of human arterial smooth muscle cells (SMCs) from patients undergoing coronary artery bypass surgery (n=11) has been reported [20–21]. The analysis by 2-DE and identification by MALDI-TOF MS yielded 83 proteins arising from SMC lysis (cellular) and 18 protein secreted into SMC conditioned medium. This was one of the first attempts [20] to identify not only the proteome expressed by cultured SMCs, a frequent model for the study of pathogenesis of atherosclerosis, but also the SMC secreted proteins, which could be related to biological markers in the circulation. The proteins secreted by SMCs and identified in this study are listed in the Supporting Information Table S1. In addition to global analysis of the secretome from SMCs, the efforts have been made to analyze particular proteins and factors and compare their expression to the levels under pathology induced conditions and other stimuli. For example, the study aimed to purify and identify secreted oxidative stress-induced factors from the secretome of vascular SMCs (VSMCs) [22] has been carried out. Upon oxidative stress induced by the naphthoquinolinedione LY83583, the proteins released into conditioned medium (2 h of LY83583 exposure) included heat shock protein HSP90- α and cyclophilin B, and HSP90- α was found to affect extracellular signal-regulated kinase (ERK1/2) activation. In recently published paper [23], vascular remodeling induced by low shear stress and cross-talk between VSMCs and endothelial cells (ECs) in co-culture system have been evaluated. The low shear stress up-regulated migration and proliferation of ECs and VSMCs increased production of platelet-derived growth factor PDGF-BB and transforming growth factor TGF- β 1, enhanced expressions of lysyl oxidase and phospho-ERK1/2, and decreased lamin A in both ECs and VSMCs. Interestingly, TGF- β 1 had similar influences on ECs as PDGF-BB, but not on VSMCs. This suggests that ECs convert the low shear stress stimulus into up-regulation of PDGF-BB and TGF- β 1 with PDGF-BB involved in the paracrine control of VSMCs by ECs, whereas TGF- β 1 participates in the feedback control from VSMCs to ECs.

2.2 Endothelial cells

Recently, study has been published in which paracrine factors originating from vascular endothelial cells facilitated cardiomyocyte differentiation of mouse embryonic stem cells (ESCs) [24]. In this study, the effect of conditioned media obtained from aortic ECs, neonatal cardiomyocytes (CMs) or embryonic fibroblasts (FBs) on ESC differentiation was examined. The conditioned media accompanied by up-regulation of cardiac-specific genes as well as other mesodermal genes were added to the ESCs differentiation medium and monitored over period of 10 days. The cardiac differentiation of ESCs was significantly increased in the conditioned medium from ECs, whereas no significant effect was observed with addition of conditioned medium of CMs or FBs. As well, the best results were obtained when EC-conditioned medium was added at an early stage (0–3 days) of ESC differentiation culture. On the other hand, the cardiomyogenesis by EC-conditioned medium was prevented by noggin, prostaglandin I and L-NAME, i.e. inhibitors of bone morphogenic protein BMP, cyclooxygenase COX, and nitric oxide synthetase NOS, respectively, suggesting the paracrine effects of BMP, COX and NOS on ESC cardiomyogenesis.

Although proteomic analysis of human umbilical vein endothelial cells (HUVECs) has been carried out [25–27], the mapping of HUVEC secretome has proved to be more challenging since HUVECs require serum supplements which mask secreted proteins in the conditioned medium [28].

2.3 Progenitor/stem cells

2.3.1 Smooth muscle progenitor cells—Recently, a study has been published that compared the secretomes of smooth muscle progenitors (SPCs) with early angiogenic cells (EPCs; endothelial progenitor cells) and human aortic SMCs by using shotgun proteomic strategy [29]. Label-free differential expression analysis has shown protein profiles distinguishing EPCs from SPCs with 19 and 46 proteins significantly increased in conditioned media of SPCs and EPCs, respectively. The conditioned medium of SPCs consisted of number of ECM proteins such as various types of collagens, ECM protein 1, fibrillin-1, fibronectin and SPARC, while the conditioned medium of EPCs contained a variety of cathepsins and platelet proteins. The SPC and aortic SMC conditioned media contained 100 proteins common to both SPCs and SMCs with 32 and 38 proteins predominantly found in conditioned medium of SPCs and SMCs, respectively. There was a significant overlap among ECM proteins present in conditioned medium of SPCs and SMCs, e.g. proteases and protease inhibitors, types of collagen (collagen α -1 (I,III,V,VI) and collagen α -2 (I,V,VI)) laminin subunits, proteoglycans and glycoproteins (fibronectin, decorin, follistatin-related protein 1 and SPARC) and insulin-like growth factor IGF-binding proteins. The proteins identified in secretomes of SMCs and SPCs [29] are included in the Supporting Information Table S1.

2.3.2 Endothelial progenitor cells—The EPCs can be obtained from peripheral blood-derived mononuclear cells [30] and it has been shown that they are involved in neovascularization [31] and angiogenesis [30]. Proteomic characterization of the proteome and secretome of human early pro-angiogenic cells (EPCs) was published recently [27] using a DIGE and shotgun MS-based approach. In [27], total 82 human proteins were identified in the conditioned medium of EPCs with MS confirmation of presence of cathepsin L, which was previously detected by gene expression profiling of EPCs and indicated as a crucial factor of postnatal neovascularization [32]. The list of proteins secreted by EPCs [27] is a part of the Supporting Information Table S1. To assess the effects of cathepsin L inhibitors on EPCs secretory potential, secreted proteins of EPCs without and with cathepsin L inhibitor treatment (EPCs incubated 3 h in serum-free medium with 10 μ M cathepsin L inhibitor) were analyzed by DIGE and LC-MS/MS. The analysis of

differentially expressed protein spots resulted in 81 protein identifications and it demonstrated the effects of cathepsin L inhibitor on EPC secretion, e.g. beta-hexosaminidase, other cathepsins, lamin-A/C and B1, thymidine phosphorylase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, and annexin A2 were down-regulated compared to control, whilst e.g. vimentin, Rho GDP-dissociation inhibitors 1 and 2, fructose-bisphosphate aldolase A and proteins S100-A8, A9 and A11 expression was stimulated by cathepsin L inhibitor treatment and the protein levels exhibited up-regulation.

2.3.3 Cardiac progenitor cells—Cardiac progenitor cells (CPCs) have been reported as a resident population of adult stem cells found in postnatal hearts [33–34]. They were identified by using various assays and they are positive for a number of stem cell markers e.g. c-Kit and Sca-1 and MDR-1 [35]. CPCs have been shown to differentiate into cardiomyocytes, ECs and SMCs in many species including humans [36–37]. Even though controversial results have been published, CPCs were found to improve cardiovascular regeneration after myocardium injury *in vivo* [35,38–40] as well as with paracrine/autocrine mechanisms involved [15,40–42]. Simultaneously, with efforts to identify CPC secretomes and possible paracrine/autocrine factors, numerous studies have been published in few recent years based on engineering of biomaterial scaffolds and sheets, with aim to improve stem cell survival rates and their functions after transplantation into damaged myocardium [43–44]. Since CPCs as well as other progenitor cells are located in tissue in spacial microenvironments (niches) which maintain and regulate them together [45] with present soluble and cell secreted factors, one can imagine, that after injury when surrounding ECM components are lost, the biomaterial can serve as ECM replacement with chemical and biophysical interactions [46–48] and could promote cell survival, function, retention and engraftment. In addition to contribution of CPCs to the repair of heart injury, differentiated cardiomyocytes have been induced to proliferate and regenerate as well [49]. In this case, neuregulin 1 (NRG1) and its tyrosine kinase receptor (ErbB4) were involved in molecular mechanism for controlling of this process. NRG1 induced mononucleated cardiomyocytes to divide and increasing ErbB4 expression *in vivo* enhanced cardiomyocytes proliferation. Thus, increase in the activity of the NRG1/ErbB4 signaling pathway might promote myocardial regeneration and improvement of the function after myocardial infarction.

Recently, our group has published a study showing distinction between secretomes of rat CPCs (analyzed as both cardiospheres and cardiosphere-derived cells [50]) and neonatal rat ventricular myocytes (NRVMs) [13]. The cells were conditioned in medium containing 1% of FBS for 48 h, pre-fractionated by RPLC and proteins were identified by LC-MS/MS. We identified 83 non-redundant proteins, of those, 49% and 23% were NRVM- and CPC-specific, respectively. Fifteen proteins fulfilled our criteria for paracrine/autocrine factors and most of these proteins have not been previously linked to stem cells. Three proteins, NRVM-specific atrial natriuretic factor (ANP) and CTGF, and CPC-specific interleukin-1 receptor-like 1 (ST2) have been further investigated and they have shown to affect rat CPC proliferation in paracrine (ANP and CTGF) and autocrine (ST2) manner.

The experimental conditions used for identifications of SMCs, SPCs, EPCs, CPCs and NRVMs [13,20,27,29] are summarized in Table 1. The compilation of identified proteins [13,20,27,29] is given in the Supporting Information Table S1. The protein identifications were compared to the identifications of the Human Plasma PeptideAtlas 2010-05 (www.PeptideAtlas.org, HPP) [51] and they can be seen in the Supporting Information Table S1 as well together with protein subcellular locations as found in the Protein Knowledgebase (UniProt KB/Swiss-Prot). In addition, Figure 2 shows the protein distribution between progenitor (EPCs, SPCs, CPCs) and adult (NRVMs, SMCs) cells as assembled from protein list in Supporting Information Table S1 [13,20,27,29]. The proteins with the same names but identified for different species were counted as one protein in the

Supporting Information Table S1 and the resulting protein was assigned to all type cells in which was detected. For example, extracellular matrix protein 1 (ECM1) was identified with 2 species assignments (rat and human), rat ECM1 was secreted by NRVMs and human ECM1 by both SMCs and SPCs. The protein was counted as one item but overlapping among these cell types (Figure 2). Thus, 123 (48%) secreted proteins were found to overlap between progenitor and adult cells from total 258 counted proteins (Figure 2, panel A). By comparison among progenitor cells, 34 secreted proteins were common between EPCs and SPCs but only 7 and 8 secreted proteins overlapped between CPCs-EPCs and CPCs-SPCs, respectively. 3 (1.5%) proteins out of total 194 proteins assigned to progenitors were common to all three progenitor cell types (Figure 2, panel B). From total 187 secreted protein by the adult cells, only 17 (9%) secreted proteins overlapped between NRVMs and SMCs (Figure 2, panel C). Based on these results, two secretomes of progenitor (EPCs, SPCs and CPCs) and adult (NRVMs and SMCs) cells share certain similarities (48%) but the secretomes are very distinct among progenitor cell themselves and between adult cell themselves as well. It is important to mention, that these results were acquired from different labs, experimental and instrumental conditions, and for different species (Table 1). Nevertheless, the secretome diversity appears to be much bigger than the cell diversity.

3 *In vivo* secretome sampling

Most secretome identifications are carried out in *in vitro* arrangements. However, the *in vivo* setting is more complex and reflects the true physiological situation. Although the ultimate goal is analysis of secreted proteins *in vivo*, the absence of appropriate technology limits this task. For example, several studies have been carried out to examine cancer secretomes *in vivo* conditions [52] including the analysis of proximal fluids such as tumor interstitial fluid which perfuses the breast tumor microenvironment [3–4] and nipple aspirate fluid containing secreted proteins from patients diagnosed with breast cancer [5–6].

Recently, a new method for *in vivo* secretome sampling was published [53–55] which has been able to dynamically collect the secretome *in vivo* from tissues and tissue fluids. The method utilized the capillary ultrafiltration (CUF) probe which can be implanted into various tissues to harvest the tissue fluids and extract fluid from the extracellular space that contains secreted and ECM proteins. The CUF probe extracts tissue fluids by applying a vacuum to semipermeable membrane fibers with different pore sizes, molecular weight cutoffs and surface charges that offer a wide range of selectivity. The technique was used for collection of secretomes directly from e.g. tumor masses, and it was linked to mass spectrometric proteomics in order to identify tumor secretome at the progressive and regressive stage [53]. Five secreted proteins (cyclophilin-A, S100A4, profilin-1, thymosin beta 4 and 10) previously associated with progression and five other secreted proteins (fetuin-A, alpha-1 antitrypsin 1 and 6, and contraptin) were detected at the progressive and the regressive stage, respectively. The advantages of CUF probe technique (no tissue damage; concentration of collected sample reflects the concentration in the tissue) were compared to other *in vivo* methods used for collecting of tissue fluids such as perfusion and saline wash (contamination with other substances from cells damaged in the sampling process; inability to collect the fluids from specific microenvironments of interest), fine-needle aspiration (contamination from blood and needle-damaged tissue) and microdialysis (slow protein diffusion through semipermeable membrane and time-consuming recovery calculations) [55].

Another method published recently is based on self-assembled peptide hydrogels [56–57] which have already shown to be promising therapeutic material serving as e.g. biocompatible cell-seeded scaffolds in damaged tissue regeneration [58–60] or local therapeutic agent administration delivery [61–62]. In [56], the multidomain peptide

nanofiber hydrogel was used as a delivery material for human embryonic stem cell (ESC) secretome. The multidomain peptide self-assembles into nanofibers in the presence of Mg^{2+} and forms cross-linked viscoelastic hydrogel which can be used as injectable material since it undergoes shear thinning with quickly elasticity recovering when shearing force is released. The peptide hydrogel acts as a sponge and soaks up the secreted proteins when placed in the presence of human ESCs and it can release the ESC secretome into a therapeutic environment upon injection *in vivo*. As benefit of stem cell therapy is indisputable but bears the limitation in clinical application since possibility of teratoma formation and risks of the rejection by the immune system, this technique can be alternative approach for delivery of secreted proteins directly to the place of need and without the problems of limited cell engraftment and viability. The method has been tested in renal recovery following acute kidney injury, and it has shown renoprotective properties of the peptide nanofibers preconditioned with ESC secretome [57].

A similar approach based on the capturing the stem cell paracrine effect using self-assembling injectable peptide nanofibers presenting heparin has been the subject of the study published recently for treatment of cardiovascular diseases [63]. The mouse mesenchymal stromal cells (MSCs) were conditioned in serum-free medium for 18 h under hypoxic conditions (5% O_2 , 5% CO_2 and 90% N_2) to mimic this stem cell paracrine effect, then conditioned medium was collected, depleted to different degrees of heparin-binding factors (by using the methods with various heparin depletion efficiencies) and partitioned into three size-based fractions (< 10 kDa, 10–30 kDa and > 30 kDa) using centrifugal filtration. The peptide nanofiber material was soaked in hypoxic conditioned medium for 24 h at 4 °C to load the stem cell secreted proteins and it was injected either into the heart following coronary artery ligation in a mouse ischemia-reperfusion model of acute MI or into a rat chronic ischemic hind limb model. In the mouse ischemia-reperfusion infarction model, significant preservation of haemodynamic function was observed with crucial secreted factors primarily < 30 kDa and primarily heparin-binding. This was re-confirmed by experiments with recombinant VEGF- and bFGF-loaded peptide nanofibers. In this case, the peptide nanofiber material served as a synthetic ECM and a biomimetic delivery vehicle for paracrine factors. In a chronic rat ischemic hind limb model, the stem cell secreted protein-loaded materials contributed to extensive limb revascularization.

Based on these experiments, the use of synthetic biomaterials which bind and deliver the needed recombinant factors to the proper locations *in vivo* appears to be a promising approach as an acellular therapy. All of these new *in vivo* collection and testing methods can be adapted to cardiovascular research in the future. On the other hand, the implantation of a foreign objects such as synthetic biomaterials could arise the questions about the inflammatory and other accompanying effects on a local tissue. Although not discussed in any aforementioned studies, these effects might influence the result of regeneration and repair process.

4 Secreted proteins as the candidate biomarkers

As proteins secreted into cell conditioned media *in vitro* can be most probably found circulating in blood/plasma *in vivo* and thus identified as the potential biomarkers for patient screening, we compared secreted proteins identified in Refs. [13,20,27,29] with the list of protein identifications of the Human Plasma PeptideAtlas 2010-05 (www.PeptideAtlas.org, HPP) [51] (see Supporting Information Table S1 and Table 2) and with two sets of proteins published in 2005 as cardiovascular-related proteins identified in human plasma by the HUPO Plasma Proteome Project Pilot Phase [64] and as candidate-based proteomics in the search for biomarkers of cardiovascular disease [65] (Table 2). Table 2 includes the subset of proteins from the Supporting Information Table S1 which were specific to only one cell

type (SMC, SPC, EPC, CPC or NRVM), they were identified as secreted or PM proteins (UniProt KB/Swiss-Prot search), and if the same protein name for different species and in different cell type secretomes was found, the protein was excluded from the list as not specific. The collagens and complement factors are not included in Table 2 even if they were cell type specific. From total 62 proteins included in Table 2, 44 proteins were seen in HPP but we found only 8 and 9 secreted proteins of Table 2 to be cardiovascular-related when compared to Refs. [64] and [65], respectively. Since progress in the protein biomarker field is moving fast, there is high number of articles dated from 2005 and reporting the studies in which various proteins have been identified as the markers. We performed extensive search on proteins in Table 2 to see if any of those proteins have been ever included in the biomarker study of cardiovascular diseases and cancer and the results are shown in last column of Table 2 together with the references. Only one representative reference for each protein and each disease type is given. The protein names as well as their synonyms given in Protein Knowledgebase (UniProt KB/Swiss-Prot) were searched in PubMed website with keywords and their combinations as follows: “protein name AND diagnostic AND biomarker AND cardiac AND ischemia/heart failure/angiogenesis” or “protein name AND diagnostic AND biomarker AND vascular AND dissection” or “protein name AND diagnostic AND biomarker AND cancer”. The search outcomes were further examined and selected. As expected, many proteins have been identified as biomarkers of cancer (28 from total 62) but 18 proteins have been shown to be tested as biomarkers in cardiovascular diseases as well. Of note, the majority of the biomarker studies (Table 2) have been carried out during period from 2006 to 2011 (37 from total 49) which demonstrates the enormous interest and progress in the field.

In addition, the secretome proteins included in the Supporting Information Table S1 (with subcellular location marked as secreted or as PM proteins by Protein Knowledgebase) were subjected to Ingenuity Pathway Analysis (IPA; 2000–2011 Ingenuity Systems, Inc.) to determine the biological pathways and functions which were most significant to those proteins. The proteins secreted by the cells were uploaded to IPA separately for each cell type (SMC, SPC, EPC, CPC or NRVM) and Figure 3 demonstrates the bar diagram of the top canonical pathways identified by IPA for secretome proteins of the Supporting Information Table S1. The number of canonical pathways was narrowed by using the criterion that at least two proteins were seen in each of three or more cell types for given canonical pathway. Among the top pathways, the canonical pathway “Acute Phase Response Signaling” was common to secretomes of SMCs, SPCs, EPCs and CPCs but not to secretome of NRVMs. Eleven secreted proteins involved in this pathway were cell specific, i.e. plasminogen (PLG), hemopexin (HPX), neutrophil defensin (HP), tissue factor (TF), fibrinogen alpha (FGA), alpha-2-macroglobulin (A2M), fibrinogen gamma (FGG), histidine-rich glycoprotein (HRG) (Table 2), and complement C1r (C1R), complement C3 (C3) and complement factor B (CFB) (Supporting Information Table S1). The biological network (graphically displayed as edges (biological relationships) between nodes (genes/ gene fragments compiled by Ingenuity Pathways Knowledge Base for uploaded secretome proteins)) of canonical pathways as analyzed by IPA for those eleven proteins is shown in the Supporting Information Figure S1. For the proteins involved in the “Acute Phase Response Signaling” pathway, the network nodes are rounded by orange circles and eleven secreted protein nodes are marked in different colors to identify cell types. The “Acute phase response signaling” is a pathway which is initialized as a response of the organism to tissue injury, infection and inflammatory processes [66]. The mediators of the acute phase response are mostly cytokines (e.g. IL-6) and one of the features of this response is the alteration in the concentration of the proteins known as acute phase proteins (APPs). It was documented that elevated levels of APPs are often found in patients with cardiovascular diseases [67]. The majority of APPs is synthesized in the liver and their functions include restoring haemostatic functions (e.g. fibrinogen), microbicidal and phagocytic functions

(e.g. complement components), antithrombotic properties (e.g. alpha-1-acid glycoprotein), and antiproteolytic actions (e.g. alpha-2-macroglobulin) [66].

Although IPA is characterized as one of the proof-of knowledge based software which comprises extensive library of well-characterized signaling and metabolic pathways and thus, it is very useful tools for understanding of the biological pathways and functions significant to examined proteins, it depends on the final goals of the study how to interpret and follow up on the results obtained from IPA. Even though the use of pathways analysis software helps to understand the protein-protein relationships without the laborious manual literature search, still the manual confirmation of the results or the custom search modification (e.g. including only recent literature or excluding the older reports) should be performed in some cases.

Concluding remarks

The cell secreted proteins proved to be a valuable source of biomarkers as they reflect actual state of the cells in real time and at particular situation such as during development, maturation and pathophysiological conditions. In particular, the cancer research field includes a high number of secretome studies and programs which have been carried out on various types of cancer [68–69] with novel secreted protein biomarker candidates detected. For example, an extensive study has been published recently [70] in which secretomes of 23 cancer cell lines derived from 11 cancer types were analyzed by LC-MS/MS and the candidates of secreted protein biomarkers indicated. By using the protein secretion-predictive algorithms, 55.8% of the proteins were confirmed to be released or shed from the cells [70].

In this review, we discussed the findings which have been published for secreted proteins identified in conditioned media of five cell types relevant to cardiovascular research. Similarly to secretomes identified in other fields, there are the difficulties involved in comparison the studies arising from various labs since various experimental conditions, methods and evaluation tools are used. On the other hand, this can be beneficial if various protein separation, validation and identification methods are used for the same cell type since they can contribute to broader proteome coverage. In the mining for potential protein biomarkers, the proper and efficient selection of the biomarker candidates from the expanding list of identified proteins is challenging. Nevertheless, since the blood and other body fluids consist of proteins cumulated from various tissues and organs (not only from the one which is of the particular interest), the identification and pre-selection of candidate protein biomarkers from the cell secretomes with subsequent validation of their presence in blood of patient during clinical screening is promising.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank to Vidya Venkatraman for Ingenuity Pathway Analysis help. Supported by the National Heart, Lung, and Blood Institute Proteomic Initiative - contract NHLBI-HV-10-05 (2) (JEV), by the AHA grant in-aid (JEV) and by the Institutional Research Plan AV0Z40310501 of the Academy of Sciences of the Czech Republic (MS).

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Figure 1.

Basic steps involved in ultimate goal to identify and characterize the secreted proteins in cell conditioned medium (the secretome) as a part of biomarker discovery.

Figure 2.

The protein distribution between progenitor (EPCs, SPCs, CPCs) and adult (NRVMs, SMCs) cells as assembled from lists of proteins in Supporting Information Table S1 [13,20,27,29]. EPCs – endothelial progenitor cells, SPCs – smooth muscle progenitor cells, CPCs – cardiac progenitor cells, NRVMs – neonatal rat ventricular myocytes, SMCs – smooth muscle cells.

Figure 3.

The bar diagram of the top canonical pathways identified by IPA for proteins secreted by SMCs, SPCs, EPCs, CPCs and NRVMs [13,20,27,29] and listed in the Supporting Information Table S1. SMCs - smooth muscle cells, SPCs – smooth muscle progenitor cells, EPCs – endothelial progenitor cells, CPCs – cardiac progenitor cells, NRVMs – neonatal rat ventricular myocytes. The description is given in the text.

Table 1

Summary of the experimental conditions and instrumentation used for identification of proteins in secretomes of SMCs, SPCs, EPCs, CPCs and NRVMs [13,20,27,29].

	Ref.[20]	Ref. [27]	Ref. [29]	Ref. [13]
cell type	arterial SMCs	EPCs	aortic SMCs, SPCs	CPCs, NRVMs
species	human	human	human	rat
cell source	internal mammary artery	peripheral blood	SPCs from peripheral blood	cardiac tissue from left ventricle
conditioning medium	serum-free	serum-free	serum-free	1% FBS
time of conditioning	24 hours	24 hours	24 hours	48 hours
preparation method and digestion	acetone-ethanol precipitation; in-gel digest of 2-DE spots	in-solution trypsin digest	in-solution trypsin digest	trypsin digest of dried RPLC fractions
separation method	2-DE	nano RPLC	nano RPLC	RPLC
mass spectrometry	MALDI-TOF	MS/MS	MS/MS	LC-MS/MS
database (species)	NCBI, Swiss-Prot (human)	UniProtKB/Swiss- Prot (human+bovine)	UniProtKB/Swiss- Prot (human+bovine)	NCBI (all species)

Table 2

Comparison of the subset of secreted proteins (from Supporting Information Table S1; [13,20,27,29]) to protein identifications of HPP [51] and cardiovascular-related protein datasets [64–65] with a literature search on biomarker studies.

protein name ¹	accession number ²	cell type ³	seen in HPP ⁴ [51]	seen in Ref. [64] ⁵	seen in Ref. [65] ⁶	linked to a biomarker study ⁷
37/67 kDa laminin receptor [R. norvegicus]	gi 730681 (P38983)	NRVMs				
Adrenomedullin [R. norvegicus]	gi 38197392	NRVMs				ischemia [71], MI [72], cancer [73]
Alpha-2-macroglobulin	A2MG_HUMAN (P01023)	EPCs	yes	yes	yes	MI in diabetes/AIDS patients [74–75]
Atriopeptin (ANP) [R. norvegicus]	gi 113870 (P01161)	NRVMs			yes	HF [76]
Carboxypeptidase E [R. norvegicus]	gi 115893 (P15087)	NRVMs				cancer [77]
Casein alphaS1 [B. taurus]	gi 225632	CPCs				prostate hyperplasia [78]
Cat eye syndrome critical region protein 1	CECR1_HUMAN (Q9NZK5)	EPCs	yes			
CD163 antigen	C163A_HUMAN (Q86VB7)	EPCs	yes			
CD59 glycoprotein (Protectin)	CD59_HUMAN (P13987)	SMCs	yes			HF [79], cancer [80]
Clusterin [R. norvegicus]	gi 461756 (P05371)	NRVMs			yes (human)	MI [81], cancer [82]
Connective tissue growth factor [R. norvegicus]	gi 11560085	NRVMs				HF [83], cancer [84]
C-type lectin domain family 11 member A	CLC11_HUMAN (Q9Y240)	SMCs	yes			
Cystatin E/M [B. taurus]	gi 61097917	CPCs				cancer [85]
Deoxyribonuclease I	DNAS1_BOVIN (P00639)	SMCs				ischemia [86], cancer [87]
Dkk3 protein [R. norvegicus]	gi 55715848	NRVMs				cancer [88]
EGF-containing fibulin-like ECM protein 1	FBLN3_HUMAN (Q12805)	SMCs	yes			cancer [89]
EGF-like repeat and discoidin I-like domain-containing protein 3	EDIL3_HUMAN (O43854)	SPCs	yes			cancer [90]
Endostatin	CD248_HUMAN(Q9HCU0)	SMCs	yes			
Fibrinogen alpha chain	FIBA_HUMAN (P02671)	EPCs	yes	yes	yes	ischemia [91], cancer [92]
Fibrinogen gamma chain	FIBG_HUMAN (P02679)	EPCs	yes	yes	yes	ischemia [91], cancer [92]
Gamma-interferon-inducible lysosomal thiol reductase	GILT_HUMAN (P13284)	EPCs	yes			
Gelsolin	GELS_HUMAN (P06396)	EPCs	yes	yes		cancer [93]
Granulins (Proepithelin)	GRN_HUMAN (P28799)	EPCs	yes			cancer [94]
Haptoglobin	HPT_HUMAN (P00738)	SMCs	yes	yes		cancer [95]

protein name ¹	accession number ²	cell type ³	seen in HPP ⁴ [51]	seen in Ref. [64] ⁵	seen in Ref. [65] ⁶	linked to a biomarker study ⁷
Hemopexin [H. sapiens]	gi11321561	CPCs		yes	yes	cancer [96]
Histidine-rich glycoprotein [H. sapiens]	gi123523 (P04196)	CPCs	yes			cancer [97]
Insulin-like growth factor-binding protein 2	IBP2_BOVIN (P13384)	SPCs				HF [98], cancer [99]
Insulin-like growth factor-binding protein 4	IBP4_HUMAN (P22692)	SMCs	yes			cancer [100]
Integrin beta-1	ITB1_HUMAN (P05556)	SMCs	yes			cancer [101]
Interleukin-1 receptor-like 1 [R. norvegicus]	gi26395279 (Q62611)	CPCs				HF [102]
Laminin subunit alpha-4	LAMA4_HUMAN (Q16363)	SMCs	yes			
Lymphocyte-specific protein 1	LSP1_HUMAN (P33241)	EPCs	yes			
Lysozyme C	LYSC_HUMAN (P61626)	EPCs	yes			cardiac atherosclerosis [103]
Macrophage inflammatory protein 2-beta	MIP2B_HUMAN (P19876)	SMCs	yes			
Monocyte differentiation antigen CD14	CD14_HUMAN (P08571)	EPCs	yes	yes		HF [104], prostatic hyperplasia [105]
Neutrophil defensin 1	DEF1_HUMAN (P59665)	EPCs	yes			cancer [106]
Osteopontin [R. norvegicus]	gi1871124	CPCs				HF [107]
Pappalysin-1	PAPPI_HUMAN (Q13219)	SMCs	yes			
Peolce protein [R. norvegicus]	gi55824687	NRVMs				
Pentraxin-related protein PTX3	PTX3_HUMAN (P26022)	SMCs	yes			
Phospholipid transfer protein	PLTP_HUMAN (P55058)	EPCs	yes			coronary heart disease [108]
Plasminogen	PLMN_BOVIN (P06868)	SPCs			yes (human)	
Platelet basic protein (PBP)	SCYB7_HUMAN (P02775)	EPCs	yes			cancer [109–110]
Platelet factor 4 (PF-4) (CXCL4)	PLF4_HUMAN (P02776)	EPCs	yes		yes	coronary angioplasty [110]
Pregnancy zone protein	PZP_HUMAN (P20742)	SPCs	yes			
Ribonuclease 4	RNAS4_HUMAN (P34096)	SMCs	yes			
Ribonuclease pancreatic (RNase 1)	RNAS1_HUMAN (P07998)	EPCs	yes			
Scavenger receptor type A [M. musculus]	gi1709140 (P30204)	CPCs				
Serrototransferrin	TRFE_BOVIN (Q29443)	SMCs				cancer [111]
Small inducible cytokine A18	CCL18_HUMAN (P55774)	EPCs	yes			atherosclerosis [112], cancer [113]
Small inducible cytokine A2	CCL2_HUMAN (P13500)	SMCs	yes		yes	HF [114]
Small inducible cytokine B5	SCYB5_HUMAN (P42830)	SMCs	yes			cancer [115]

protein name ¹	accession number ²	cell type ³	seen in HPP ⁴ [51]	seen in Ref. [64] ⁵	seen in Ref. [65] ⁶	linked to a biomarker study ⁷
Small inducible cytokine B6	SCYB6_HUMAN (P80162)	SMCs	yes			
Stanniocalcin-1 (STC-1)	STC1_HUMAN (P52823)	SMCs	yes			cancer [116]
Sulphydryl oxidase 1 (Quiescin Q6)	QSCN6_HUMAN (O00391)	SMCs	yes			
Sushi repeat-containing protein SRPX	SRPX_HUMAN (P78539)	SMCs	yes			
Testican-1 (Protein SPOCK)	TICN1_HUMAN (Q08629)	SMCs	yes			cancer [117]
Thrombospondin-2	TSP2_HUMAN (P35442)	SMCs	yes	yes		
Tissue factor pathway inhibitor 2 (TFPI-2)	TFPI2_HUMAN (P48307)	SMCs	yes			cancer [118]
Transferrin receptor protein 1	TFR1_HUMAN (P02786)	SMCs	yes			cancer [119]
Versican core protein	CSPG2_HUMAN (P13611)	SMCs	yes			
V-set and Ig domain-containing protein 4	VSIG4_HUMAN (Q9Y279)	EPCs	yes			

¹ species are given in parenthesis if it is not evident from the accession number

² accession numbers as reported in the original papers. The UniProtKB accession number (e.g. P38983) is provided as well if found for particular protein in Protein Knowledgebase

³ cell type specificity for particular protein as assembled from Supporting Information Table S1

⁴ protein listed in Human Plasma PeptideAtlas 2010-05 (www.PeptideAtlas.org)

⁵ cardiovascular-related protein identified in human plasma by the HUPO Plasma Proteome Project Pilot Phase [64]

⁶ candidate biomarker protein with reported association to cardiovascular disease and stroke [65]

⁷ protein identified as a disease marker in biomarker study as assembled through literature search (PubMed)

MI - myocardial infarction; HF - heart failure; ischemia - cardiac ischemia