

Theme Issue Article

Stress testing at the cellular and molecular level to unravel cellular dysfunction and growth factor signal transduction defects: What Molecular Cell Biology can learn from Cardiology

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Summary

Clinical medicine has been revolutionized by the impact of cellular and molecular biology in the past 30 years. This article focuses on a novel approach, whereby the clinically proven and important concept of patient or organ stress testing is being applied to cellular models, thereby developing and validating novel quantitative molecular and cellular stress tests. One example is monocyte chemotaxis analysis, whereby circulating monocytes freshly isolated from peripheral blood are being tested for their migratory responsiveness towards relevant biological stimuli such as growth factors or chemokines. These stimuli are relevant for recruiting monocytes to sites of local inflammation such as during wound healing or arteriogenesis, i.e. growth of collateral arteries. Initial clinical studies to validate "ligand-induced monocyte chemotaxis" indicate that this parameter is impaired in the

presence of various cardiovascular risk factors including diabetes mellitus, hypercholesterolemia or smoking. In addition, there is proof of concept that impaired monocyte chemotaxis is reversible as shown for anti-oxidants in smokers. Moreover, the parameter "ligand-induced monocyte chemotaxis" is of great relevance for basic science (including Molecular Cell Biology) as unravelling the underlying molecular mechanisms of cellular dysfunction will certainly stimulate our understanding of the molecular basis of cellular function. This article highlights the concept of stress testing in modern medicine. Cellular stress testing is introduced as a novel and intriguing approach, which was developed as bedside-to-bench. Future prospective clinical trials will have to validate the predictive value of cellular stress testing.

Keywords

Monocytes, chemotaxis, functional cell testing, functional cellular diagnostics, stress test for vascular cells

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From bedside to bench: An example of the 21st century

Over the past three decades, clinical medicine has learned a lot from cellular and molecular biology. The list of medical innovations and progress in our understanding of the pathogenesis of disease is long, and clinical medicine has greatly benefited from the introduction of molecular and cellular concepts. Along with these innovations, clinicians have learned a great deal about the concepts of molecular cell biology. At the same time, there was reverse flow of innovation, i.e. from bedside to bench, when animal models were set up to serve as in-vivo models of clinical conditions such as mouse or rat models of hyperglycaemia and diabetes mellitus (1).

Recently, our laboratory introduced a novel "bench to bedside" approach, which focuses on stress testing of circulating cells to provide additional and valuable information about the functional status of vascular cells. Clinical medicine has learned in the past that an individual patient (just like an individual cell) may carry a fully normal phenotype under resting conditions. Under certain conditions of "stress", however, a pseudo-normal phenotype can be unmasked and be recognized as a pathological phenotype, thereby clearly proving the dysfunction of an organ system. In this context, stress can be defined as a forced physiologic response using a defined and standardized physiological or supra-physiological stimulus. Several clinical disciplines take advantage of the concept of stress testing, with Internal Medicine and Cardiology being good examples. Exercise stress testing in Cardiology (2) is the most prominent one. Other important

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Table 1: Functional assessment of circulating cells.

	Test assay, cellular parameter
Cell number	FACS (ISHAGE guidelines)
	other FACS measurements (non-standardized)
	purification of individual cell populations
	counting chamber
	colony formation
Cell function	migration
	adhesion
	proliferation
	colony formation
	differentiation potential
	gene expression

examples would be metabolic stress testing (e.g. oral glucose tolerance test) (3) or other endocrine provocation tests.

Stress testing in patients

Exercise stress testing was introduced into clinical practice only about 40 years ago (4) to predict limitations of the exercise capacity, to quantitatively assess either coronary or cardiovascular insufficiency and to detect coronary artery disease. Under resting conditions, the circulatory system uses only about 20% of its maximal functional capacity. Under conditions of maximal physical exercise, all reserves are being recruited and the efficiency of the system improves dramatically, i.e. about five-fold. Any limitation of the exercise tolerance reflects a reduced or sub-optimal function of the organ system that could not be detected under resting conditions. The exercise stress test is used to determine the function of heart and lungs by detecting:

- 1) Specific subjective symptoms of limited functionality (e.g. angina pectoris, dyspnoea).
- 2) Specific objective measures for cardiopulmonary function (e.g. pulse-pressure product, maximal performance, anaerobic threshold etc.).
- 3) Monitoring of treatment in the case of limited function.

Table 2: VEGF-dependent endothelial function and endothelial dysfunction. Aspects of endothelial function and conditions related with endothelial dysfunction are listed.

Functional aspect of circulating endothelial precursor cells
– release from bone marrow
– ability to home in target tissues
Functional aspect of endothelial cells
– ability to proliferate
– ability to migrate
– providing antithrombotic surface
– maintaining antiapoptotic status
Paracrine control of other cells
– antiproliferative for SMCs
– regulation of vascular permeability

Typical diseases that can be detected by exercise stress testing (using treadmill or bicycle) are i) reduced systolic or diastolic cardiac function as seen in cardiomyopathy or hypertensive heart disease (presenting with limited exercise capacity and dyspnoea), or ii) reduced exercise capacity secondary to exercise-related regional myocardial ischemia as seen in coronary insufficiency (coronary heart disease with one or more hemodynamically relevant coronary artery stenoses), which is usually presenting as angina pectoris or its equivalents.

An alternative to exercise stress testing is pharmacological stress testing using positive chronotropic agents such as dobutamine and atropine. Pharmacological stress testing is usually combined with imaging techniques to either detect stress-related regional perfusion defects (Tc-99m cardiac SPECT perfusion scan, stress-magnetic resonance imaging) or to detect stress-related impairment of regional myocardial function (stress-echocardiography).

Besides exercise stress testing to check the cardiovascular system, metabolic stress tests are being widely used in clinical medicine. The oral glucose tolerance test is such an example: a defined amount of glucose is given to a patient to test whether glycemic control is intact. Readout is the glucose level at a certain time point following glucose stress. This is a simple method for the early detection of prediabetes.

Cellular stress testing: A novel approach

Over the past two decades, Molecular Cell Biology has accumulated important and valuable molecular and cellular concepts, which are now available for clinical testing. This includes the establishment of disease-relevant molecular systems for cellular stimulation such as vascular endothelial growth factor (VEGF) receptor activity (5, 6) and functionally relevant cell types such as endothelial cells and monocytes (7).

Cells can be isolated from the peripheral blood of a patient and can be tested in various ways (Table 1). The standard analysis of blood-derived cells is the exact characterization using surface molecules (e.g. guidelines of the International Society of Hematotherapy and Graft Engineering, [ISHAGE]) (8) and the assessment of the cell number (per volume) of selected cell populations. Because circulating cells are fully exposed to the blood stream and therefore to all toxic and metabolic influences, they should be able to sense all negative influences exactly as endothelial cells in the vessel wall can, i.e. integrating and possibly reflecting several or even all metabolic and toxic influences. In order to detect latent and minor changes of cellular dysfunction, it appears to be reasonable to subject these circulating cells to certain physiologic stimuli, i.e. stress. Therefore, cellular stress testing may offer additional diagnostic information compared to the analysis of resting (non-stimulated) cells.

Monocytes are an important component in the process of arteriogenesis (9) as they serve as bioreactors and reservoirs of various cytokines and chemokines, which can stimulate arteriolar growth in a paracrine fashion. The VEGF system, among others, can mediate arteriogenesis (10). Besides controlling monocyte function, VEGF has been established as an important regulator of endothelial function. The most important VEGF-regulated endothelial activities that contribute to the normal en-

endothelial function are presented in Table 2. Likewise, endothelial dysfunction (11) is related to a defect in endothelial responsiveness, whereby virtually all VEGF-mediated endothelial functions are impaired (12) (Table 2). When it comes to diagnostic testing, endothelial cells have a major disadvantage, which is their localization inside the vasculature. Endothelial function can therefore only be studied by indirect methods such as acetylcholine infusion and subsequent angiography (13). In the clinical setting, endothelial cell function cannot be studied *ex vivo*. This major disadvantage, however, does not apply to circulating cells such as monocytes, which makes them so attractive for functional cellular testing.

Together with the availability of a method to quantitatively assess monocyte migration (see next paragraph “*Exercise physiology of cell populations*”), this concept offers novel perspectives and opportunities for studying the molecular basis of the biological process underlying arteriogenesis including disease-related disturbances (bedside to bench approach). The concept of ex-vivo assessment of monocyte function as a predictor of arteriogenesis in a pathological environment was first reported at the spring meeting of the German Cardiac Society in Mannheim in 1999 (14).

The action of VEGF and other homing factors can be tested on circulating monocytes *ex vivo*, based on i) the functional importance of these cells, and ii) the availability of a relevant test assay (chemotaxis analysis). Experimentally speaking, this means testing of the growth factor / chemokine pathway associated with cellular movement (15). What clinical medicine has developed for the functional testing of patients or certain organ systems can now be translated onto the cellular level to i) use the cellular assay for extending the spectrum of clinical testing, and ii) expand the knowledge of molecular cell biology to better understand cell function under stress conditions. A comparison between stress testing of humans and cells is provided in Table 3.

The whole concept of functional testing of blood-derived cells is of course not limited to monocytes. In fact, any type of circulating cell can potentially be tested depending on the cellular response to a given stimulus and depending on the abundance of these cells for functional testing. Besides monocytes, other attractive cell types to be functionally studied are lymphocytes, granulocytes (16) and progenitor cells (17). Circulating progenitor cells are currently of special interest as they are likely to contribute to vascular healing (18) and as their abundance can be used as a prognostic marker for coronary artery disease (19).

Besides chemotaxis analysis, several other functional tests allow the quantification of the cellular response following growth factor stimulation as well. Examples of such stress tests are: adhesion assay, proliferation assay, stress-induced (e.g. hypoxia-induced) gene expression analysis, the assessment of activation (e.g. tyrosine phosphorylation) of selected and functionally relevant proteins. All these functional tests are based on assessing the activation pattern of functional molecular or subcellular systems that are associated with a certain cellular response. Such responses may either indicate or reflect the functional integrity of the cell as such (i.e. the test is not negatively affected by pathological conditions) or they may represent specific cellular responses associated with specific functions (e.g. migratory responsiveness) reflecting an important functional and pathophysiologically relevant response of a monocyte or progenitor cell.

“Exercise physiology of cell populations”

Cellular stress testing can be performed in a classical manner using the modified Boyden chamber (20). The initial step in performing cellular exercise testing is to isolate certain cellular (sub)-populations without (pre)activating these cells during the isolation process. One suitable method consists of two parts: First, to isolate the white blood cell fraction (buffy coat) from whole blood using gradient centrifugation. Second, the negative or positive selection of white blood cell subsets (e.g. monocytes) out of the buffy coat using a panel of labelled antibodies that can be used to remove T cells, B cells and other cell types including CD14-positive monocytes and certain precursor cells (e.g. CD133+ cells) (17).

In the next step, the isolated cell fraction is tested for their chemotactic response. A variety of chemotactic assays is currently available. They are based on the principle initially reported by Boyden (20). Cells are suspended in a solution, placed above a filter membrane and are allowed to migrate through the (defined) pores of the membrane. After a certain time period, the cells that migrate through the membranous pores towards a gradient of the chemoattractant are subsequently counted at the basal aspect of the membrane. Alternatively, the leading front method has successfully been applied measuring the relative distance that the fastest moving cells migrated into a filter, which itself is about 100 μm thick (16). The modified Boyden chamber is commercially available and allows cellular stress-testing in a routine setting in dedicated laboratories (21).

The latest methodological perspective is to assess cellular migration in a miniaturized micro-chamber, an approach that has also been classified as “cells on the chip” (22). This approach has been demonstrated to work for granulocytes, while it remains to be demonstrated for monocytes and circulating precursor cells. As soon as such a potentially powerful method can be reliably applied to the routine assessment of monocyte function in the clinical situation, the prognostic value of monocyte chemotactic responsiveness could be tested in large clinical cohorts and thereby validated for its prognostic value.

Table 3: Stress testing of humans and cells: A comparison.

	Clinical exercise test	Cell migration test
Subject studied	Whole patient	Isolated cells (e.g. monocytes)
Exercise device	Treadmill, bicycle	chemotaxis chamber
Parameter to assess	Exercise tolerance Exercise capacity	Functional responsiveness Chemotactic capacity
Defect to be detected	Identify functional defect or exercise-related deficit	Identify functional defect or stimulation-related deficit
Monitoring therapy	Possible Valid	Possible Not validated yet
Predictive value of test	Predictive for prognosis of patient	Not validated yet

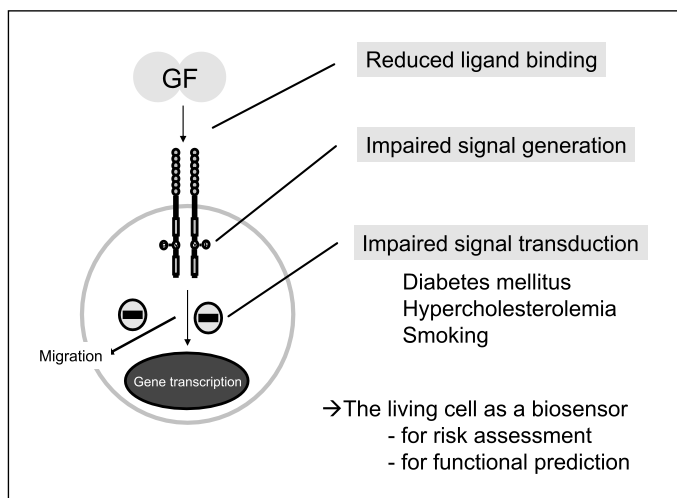


Figure 1: Cardiovascular risk and growth factor dysfunction as assessed in a living cell isolated from the peripheral blood. Various cardiovascular risk factors can induce a growth factor/related signal transduction defect, which is resulting in a migratory defect of the cells. This can be quantitatively assessed in the modified Boyden chamber. Such circulating cells may therefore be regarded as living biosensors that are capable of integrating various negative signals at different levels. GF: growth factor.

The added value of functional testing in the clinical situation is potentially large. The functional data that can be generated this way may otherwise not become available. Our group has systematically performed monocyte chemotaxis analysis in recent years. We could describe the functional response of monocytes derived from diabetic individuals (21): The migratory response of isolated monocytes from diabetic individuals towards VEGF-receptor-1 ligands (VEGF-A, PlGF-1) is significantly impaired (21), while the fMLP-induced and G-protein coupled receptor-mediated migration was fully intact and similar to the one observed in monocytes from healthy individuals (21). Likewise, hypercholesterolemia (23) and smoking (24) are associated with a functional defect in monocytes (12) that can be unmasked by assessing monocyte function in a chemotaxis assay (Fig. 1).

Molecular mechanisms causing cellular (monocyte) migration defects

What is the molecular basis of a monocyte migration defect in specific and of a reduced migratory response in general? In the search for an explanation, we investigated the cellular activation pattern on different molecular levels (Fig. 1), namely i) on the receptor level (VEGFR-1 activation), ii) on the level of relevant signal transduction molecules (e.g. activation of ERK-1), and iii) on the level of the cellular response (e.g. chemotaxis). These studies resulted in postulating cardiovascular risk factor-related intracellular signal transduction defects inside the monocyte (12). Following VEGF stimulation, there was no inhibition of VEGFR-1 activation in monocytes from diabetic patients, while VEGF-induced migration was highly reduced (21). Of significance, both cells from diabetic individuals as well as those from

healthy individuals showed a clear response to VEGF stimulation *in vitro*, namely that VEGF-A could induce tyrosine phosphorylation when tested by *in-vitro* kinase assay. This clearly indicates that the VEGF-receptor kinase is fully intact even in monocytes from diabetic individuals. The only reasonable explanation compatible with these findings is the presence of a signal transduction defect downstream of VEGF receptor-1 in monocytes from diabetic patients.

Recent data obtained in smokers indicate a signal transduction defect downstream of VEGFR-1 as well; VEGF-induced VEGFR-1 activation remained intact, while migration was severely affected (24). Like diabetic monocytes, monocytes isolated from young and otherwise healthy smokers showed a significant impairment of VEGF-induced chemotaxis, while VEGF-A was capable of inducing tyrosine phosphorylation in these cells. We could demonstrate that smoking-related monocyte dysfunction was based on the negative influence of reactive oxygen species (ROS), as a two-week oral treatment with vitamin C (1 gram twice a day) resulted in a complete reversal of the pathological monocyte phenotype. Therefore, anti-ROS treatment of smokers resulted in fully functional monocytes that were capable of properly responding to VEGF-A stimulation in a chemotaxis assay.

Cellular stress testing: Is “ligand-induced chemotaxis of circulating cells” a novel marker with prognostic relevance in the clinical situation?

The novel functional cellular parameter “ligand-induced chemotaxis of circulating cells” requires a prognostic validation in the clinical setting. Moreover, this test deserves to be validated for monitoring therapeutic interventions such as primary and secondary prevention of patients with cardiovascular risk factors. The ligand-stimulated chemotaxis assay has several potential advantages over currently available diagnostic tools: i) It provides additional information currently not assessed using other markers for risk stratification (25). ii) The test assesses a true functional parameter. iii) Monocyte (dys)function potentially reflects endothelial cell (dys)function. iv) The test is a non-invasive simple blood test that allows the assessment of an individual parameter in an objective and patient-independent manner.

The key idea behind the novel approach is to subject freshly isolated cells (monocytes) to a functional test *ex vivo*. The cell is taken out of the potentially pathological milieu, where it is subject to all potential negative influences that may negatively affect its function, namely hyperglycemia, oxidative stress (smoking, hypercholesterolemia), enhanced shear stress (hypertension) and others. The isolated cells are capable of integrating all negative and positive influences on cell function. These influences affect the outcome of the cellular stress test. One may regard these cells as living biosensors as their functional phenotype is dependent on the negative and positive influences they sense and integrate. There is no reason why the integration of negative and positive influences should differ from one vascular cell type to another one: Monocytes as well as endothelial cells are in direct contact with blood components, they are localized in close proximity to each other and are therefore subjected to the very same negative and positive influences *in vivo*. In addition,

endothelial cells and monocytes share several signalling pathways involved in migration (own, unpublished data). When subjecting living biosensors to a functional test *ex vivo*, the functional read-out should not be dependent on surrogate parameters, but on an integrated signal that reflects the functional condition of the isolated cell.

Moreover, based on the availability of a method to quantitatively assess monocyte migration, this concept offers novel perspectives and opportunities for studying the molecular basis of the biological process underlying cellular and vascular dysfunction (bedside-to-bench approach). Currently, the major limitation of quantitative cellular migration analysis is the lack of a robust routine device to assess this parameter in an easy and reliable fashion and within a reasonable period of time. Isolating the relevant cellular subsets and performing the chemotaxis analysis including quantitative evaluation is labour-intensive and time-consuming. It is therefore important to automatize and standardize this test. This would improve its feasibility and acceptance and would reduce the potential variability of the results, as the quality and reproducibility of the chemotaxis analysis is currently dependent on the experience of the laboratory and technician performing the test. Automation of the assessment would reduce the time required for the analysis. In addition, miniaturizing the assay would allow smaller blood volumes to be

studied for obtaining reliable functional cellular data. This will open novel perspectives for the routine assessment of individual cellular function. Moreover, the availability of such a test may represent an important addition to our current armamentarium for assessing individual risk and prognosis.

In conclusion, cellular stress testing represents a novel bedside-to-bench approach, in which circulating cells are subjected to functional testing. This provides additional, valuable information on the functional status of vascular cells, leading to the development of novel diagnostic tests, potentially allowing individual risk assessment, determining prognosis and optimizing therapy.

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Definitions

Stress: forced physiologic response using a defined and standardized physiological or supra-physiological stimulus.

Biosensor: Living cells whose functional phenotype is dependent on the negative and positive influences (hyperglycemia, oxidative stress, enhanced shear stress) they sense and integrate.

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