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Mechanism of NSF: New evidence challenging the prevailing theory

Ben B. Newton, PhD
GE Healthcare, Medical Diagnostics Discovery Research

Sergio A. Jimenez, MD
Thomas Jefferson University

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Ben B. Newton PhD* and Sergio A. Jimenez MD

*Corresponding author
Ben Newton PhD
GE Healthcare,
Medical Diagnostics Discovery Research,
White Lion Road,
Amersham,
HP7 9LL,
UK.
Telephone: +44 1494 543706
Mobile: +44 7796 000717
Fax: +44 1494 543284
ben.newton@ge.com

Sergio A. Jimenez MD
Jefferson Institute of Molecular Medicine,
Jefferson Medical College,
Thomas Jefferson University,

233 South 10th Street Philadelphia,
PA,
USA.

Abstract

NSF has been reported to be associated with the administration of gadolinium based contrast agents in patients with severely impaired renal function (SIRF), end stage renal disease (ESRD) or acute renal failure (ARF). Since the vast majority of these patients do not get NSF, it is highly likely that patient factors play a role in its development. Although “free" or dechelated Gd^{3+} is thought by some to be the only trigger of NSF, recent evidence suggests that chelated-Gd^{3+} may be important. Chelated-Gd^{3+} Omniscan (Gadodiamide) and Magnevist (Gadopentetate) can directly stimulate macrophages and monocytes to release profibrotic cytokines and growth factors capable of initiating and supporting the tissue fibrosis that is characteristic of NSF. In addition, an effect of chelated-Gd^{3+} on Fibroblasts has also been demonstrated. Chelated-Gd^{3+} in the form of Omniscan, Magnevist, Multihance, and Prohance increased proliferation of human dermal fibroblasts. Indeed increased numbers of Macrophages, together with activated Fibroblasts and Fibrocytes are essential cells in the fibrotic process and are present in NSF skin. Accordingly it is important that chelated-Gd^{3+}, in combination with patient cofactors, is considered in the aetiology of NSF associated with enhanced scans.

Key Words

Gadolinium Based Contrast Agent

gadolinium

chelated- Gd^{3+}

Gd^{3+}

stability

nephrogenic systemic fibrosis

severely impaired renal function

end stage renal disease

acute renal failure

oedema

inflammation

fibrosis

macrophage

phagocytosis

monocyte

fibroblast

Nephrogenic systemic fibrosis (NSF) is a rare, but potentially serious, acquired systemic disease initially described as a form of scleromyxedema (1). To date, it has only been reported in patients with severely impaired renal function (SIRF), end stage renal disease (ESRD) and those in acute renal failure (ARF) (2, 3, 4, 5). The many risk factors shown to be associated with NSF include: oedema (6), metabolic acidosis, thrombotic events, high dose erythropoietin (EPO) (7, 8), systemic inflammation (9), recent surgery, kidney disease (10) and Gadolinium (Gd^{3+}) Based Contrast Agent (GBCA) exposure (11, 12) especially when used at relatively high dose GBCA (13, 5). One current hypothesis assumes that the increased retention of GBCA, brought about by SIRF and ESRD, leads to the increased Gd^{3+} release from GBCA. This “Free" or dechelated Gd^{3+} is then postulated to trigger NSF (14). Although there is no evidence that inorganic Gd^{3+}-species directly trigger NSF, the hypothesis that dechelated inorganic Gd^{3+} might cause NSF is based on the following rationale:

1. The elimination half-life of gadolinium chelates increases in normal human volunteers from 1.5 hours to over 30 hours in patients with renal insufficiency (15, 16, 17, 18).
2. Differences in *in vitro* stability constants control the rate and extent of Gd^{3+} release (14).
3. Released Gadolinium precipitates and is phagocytosed by tissue macrophages and Gd^{3+} has been identified in the skin of affected patients (19, 20).
4. The phagocytosis of Gadolinium triggers inflammatory and fibrotic responses at the site of precipitation.
5. Detection of Gadolinium deposits in NSF tissue.

These ideas, coupled with *in vitro* stability constant parameters, have led investigators to propose that "free" gadolinium liberated from the chelate is the culprit species in NSF-associated with enhanced scans. There are several published studies that have assessed the different stability constants of GBCAs using a number of *in vitro* and *in vivo* assays and, depending on the experimental conditions and methods of gadolinium detection, these have shown great variation in both absolute values, and in relation to each other (21). It can be argued that these, and a number of other potential hypotheses based on comparative stability, have arisen, at least in part, in order to explain the difference in reported NSF case numbers between GBCA, such that agents with lower *in vitro* stability are more likely to trigger NSF. However this theory does not take into account other factors which can explain the differences in reported numbers and is largely based on thermodynamic stability (K_{therm}), a measurement made at pH 11, which does not reflect physiological conditions (see Table 1).

When taken together these data do not provide a cause and effect relationship *in vivo*. Furthermore, the observations that only a small minority of ESRD patients develop NSF (2, 5) clearly indicate that other associated factors play a significant role in the development of NSF:

Gadolinium is usually, but not always found in NSF patient biopsies (20).

Some countries have a relatively high incidence of NSF (23), while others using the same agent at standard dose have few reported cases (22).

Although there are clear differences in K_{therm} between certain agents, stability constants are more similar when the constant reflects a more physiological environment (24, 25). Table 2

shows the conditional stability constants (K_{cond}), which reflect the calculated stability at a pH of 7.4.

NSF, which was first recognized by Cowper (1) and linked to GBCA in 2006 (24), is characterized by induration, thickening and tightening of the skin. Distal parts of the body are usually most affected (26) but the trunk as well as internal organs such as the lungs, heart, liver, kidneys, skeletal muscle and diaphragm can also develop lesions. Histopathologically, NSF is characterized in early stages by the presence of dermal collagen interspersed with fluid oedema, mucin and elastin (27). Fibocytes or "spindle" cells are abundant and very closely associated with the developing fibrosis. Fibrocytes are a new class of bone marrow derived leukocyte subpopulation which display a distinctive phenotype with surface expression of procollagen, vimentin and CD34 and are capable of specifically entering and localizing to tissue injury sites (28).

Although fibrocytes are thought to account for as many as 10% of cells that are recruited to sites of acute tissue injury (28) it is not known whether these cells initiate or support the development of the fibrotic lesions associated with NSF. However the fact that fibrocytes possess the ability endocytose material in their surroundings has been proposed as a mechanism for their involvement in NSF (29). Indeed, whilst an initiator role for the fibrocyte is uncertain, it is likely that its involvement increases as disease develops. Although both CD34 and procollagen I are key markers of fibrocytes, collagen and procollagen I positivity are low in early stages. As disease develops, extracellular collagen deposition appears to correlate with increases in the procollagen I positivity of fibrocytes (27).

Fibroblasts however, being resident interstitial cells, regulate responses to local foreign material and are thus likely to initiate any response to high level exposure of GBCA in the oedematous periphery. Indeed fibroblasts are also present in early NSF disease and numbers increase steadily with lesion age (30). α-Smooth muscle actin (α-SMA) positive myofibroblasts, or "activated" fibroblasts, become increasingly frequent and florid as lesions develop, to the extent that lesions resemble a site of acute tissue injury. Myofibroblasts are abundant in the deep dermis and around subcutaneous tissues, colocalising exactly with developing fibrosis (1, 30). In fact areas rich in activated fibroblasts stain very strongly with Alcian blue which indicate elevated deposition of hyaluronan and sulfated glycosaminoglycans (31). Such data strongly implicate fibroblasts as effector cells in the development of NSF.

Macrophages are also present in NSF tissue (30, 32). Increased numbers of macrophages, increased expression of Factor XIIIa+ and TGFβ1, together with the activated fibroblasts and infiltrated fibrocytes are present in dermal and sub-dermal skin. These can also extend into the muscles along tracts of fibrotic tissue and cause a severe infiltrative myopathy in NSF patients (33). Since it has already been established that CD68+/factor XIIIa+ dendritic cells and TGFβ1 orchestrate the host response to eliminate noxious putative etiologic agents, it is likely that these factors have this role in NSF (32). The high levels of TGFβ1 expression and its role in the regulation of dendritic cell maturation (34, 35) suggest at least one mechanism that may promote lesion formation in NSF (32).

Gd^{3+}-species have also been found in NSF tissue co-localised in areas of dermal inflammation rich in macrophages. In freshly cut paraffin block surfaces gadolinium was detected ranging from 1 to 2270 cps/mm2 in 57 cutaneous biopsies of NSF. Gd was associated with P, Ca, and usually Na in tissue deposits (19). High *et al.* analyzed thirteen affected skin samples from seven patients (20). Gadolinium had been deposited in four of thirteen samples (four of the seven patients with NSF) at an average concentration of 70 ppm. A sample of uninvolved skin (with actinic keratosis) from a patient with NSF had a gadolinium concentration of only 5 ppm. High *et al* (20) detected insoluble Gd^{3+}-species of $<1\mu m$ in diameter confined to areas of fibrosis in NSF tissue. Since Gd^{3+}-species was considered to be intracellularly located, possibly within lysosomes, it was proposed that NSF was caused by precipitated Gd^{3+}-species being phagocytosed by macrophages which in turn produce and secrete the cytokines and growth factors that stimulate fibrosis (20).

Whilst the observations by Thakral, Abraham and High are extremely important in our understanding of the disease, the reasoning currently offered to explain the presence of Gd^{3+}-species in NSF tissue may not necessarily be correct. The basis for this assumption is that "free" Gd^{3+}, which can be toxic to cells, is suspected to be the causative agent. Indeed the toxicity of Gd^{3+} necessitated chelation so that it could be safely used as an MR imaging agent. Chelation both shields the body from damage and promotes GBCA rapid elimination. However, there is now emerging alternative evidence to suggest that even chelated-Gd^{3+} has the ability to initiate cellular processes that were previously attributed to precipitated Gd^{3+} alone.

It has now been shown that chelated-Gd^{3+} (Gadodiamide (Gd^{3+}-DTPA-BMA, Omniscan) and Gadopentetate dimeglumine (Gd^{3+}-DTPA, Magnevist)) can directly stimulate human monocytes and macrophages to release profibrotic cytokines and growth factors capable of initiating and supporting the tissue fibrosis that is characteristic of NSF (36). In these studies human peripheral blood monocytes were exposed to varying concentrations of GBCA and $GdCl_3$. Changes in expression levels of relevant cytokines and growth factors were assessed by real-time polymerase chain reaction (rt PCR) of RNA isolated from peripheral blood monocytes and by quantitative assessment of numerous cytokines, growth factors, and other inflammatory mediators employing Searchlight ELISA of culture media. Not surprisingly $GdCl_3$ was shown to activate the expression of IL-6, IL-13, TGF-β and VEGF mRNA by normal human peripheral blood monocytes. However, what was unexpected was the activation of cytokine release by Gadodiamide and Gadopentetate dimeglumine. Cytokine levels assayed in culture supernatants from the treated cells by Searchlight ELISA confirmed the increased secretion of profibrotic cytokines and growth factors to chelated-Gd^{3+}. Such studies provide a direct mechanistic link with the histopathological findings of Thakral and Abraham (19) and High (20).

In a series of follow-up studies Del Galdo (37) exposed terminally differentiated human peripheral blood macrophages to either Omniscan or saline before isolating and analysing cellular RNA for global gene expression microarray analysis. Volcano plot analysis of the microarray data revealed that 31 genes were up-regulated by more than two-fold in the Omniscan treated macrophages ($p<0.05$). Pathway analysis and rt PCR validation of the up-regulated genes strongly suggested the participation of Toll-Like Receptor (TLR) mediated activation and immunofluorescence analysis of activated cells demonstrated NFκB translocation from the

cytosol to the nucleus within minutes of GBCA application. Indeed 5 out of 9 genes were chemokine genes from the CC and the CXC families. ELISA of culture supernatants from cultured macrophages exposed to Omniscan indicated that CCL2, CCL8, CXCL10 and CXCL11 were up-regulated between 10 and 240 fold higher than saline controls. Correspondingly, immunofluorescence analysis of NSF skin biopsies revealed that CCL8 (MCP-2) was preferentially up-regulated compared to normal skin. Such observations support the mechanistic relevance of the *in vitro* studies.

Similarly, a direct effect of chelated-Gd^{3+} has also been demonstrated on fibroblasts *in vitro*. In a study by Edward (38), gadodiamide added to culture medium, stimulated fibroblast growth. In the same study, fibroblasts exposed to gadodiamide synthesized increased levels of hyaluronan. NSF Fibroblasts cultured from the lesions of six NSF patients, all of whom were exposed to gadodiamide, synthesized excess levels of hyaluronan and collagen compared to control fibroblasts. In a separate study (39) chelated-Gd^{3+} in the form of Omniscan, Magnevist, Multihance, and Prohance all increased proliferation of human dermal fibroblasts in monolayer culture. Additionally, increased proliferation of cells was accompanied by an increase in production of Matrix Metalloproteinase-1 (MMP-1) and Tissue inhibitor of Matrix Metalloproteinase-1 (TIMP-1) but no increase in type I procollagen (39,40). Specific analysis of the signaling events immediately after GBCA application shows activation of tyrosine kinases within minutes following GBCA exposure, again suggesting that chelated-Gd^{3+} could be acting as the trigger molecule (40).

Once again such studies provide a direct mechanistic link with the histopathological findings of Neudecker (31) and Jimenez (32) in NSF. Perhaps as important, NSF patient serum stimulated control skin fibroblasts *in vitro* whilst healthy control serum was without effect (38). This signifies that substances other than GBCA e.g. proinflammatory and or profibrotic cytokines, are secreted into serum by monocytes and or macrophages after GBCA exposure, and could control, participate or modulate the pathogenesis of NSF.

These observations are extremely important since Wermuth (36) has demonstrated that conditioned media isolated from GBCA-exposed PBMC caused cultured human dermal fibroblasts to increase expression of extracellular matrix proteins and α-SMA indicating their conversion into myofibroblasts (36). Since these studies show that it is possible for products of inflammatory cells to induce a fibrotic phenotype in normal dermal human fibroblasts *in vitro*, it is not inconceivable that GBCA, under certain conditions, could stimulate inflammatory cells to secrete the mediators essential to the development of NSF *in vivo*. It is interesting to note that all but one of the six samples studied in the Edward experiments came from NSF patients with higher than normal levels of inflammatory cells; because of a pre-existing inflammatory condition at the time of their enhanced scans (38).

This emerging evidence permits an alternative hypothesis (Figure 1):

1. Protracted retention of GBCA in renal insufficiency provides the conditions for enhanced exposure of tissues to GBCA.

2. GBCA could themselves trigger inflammatory and fibrotic responses in the tissues of susceptible cells.
3. GBCA interacting with cells may be internalised via receptor driven phagocytosis in macrophages and receptor mediated endocytosis in fibroblastic cells.
4. The highly acidic environment inside lysosomes could provide the conditions for dechelation of GBCA.

It is important to point out that receptor-mediated endocytosis of chelated Gd^{3+} has been proposed previously (41). Franano observed acid dependent metabolism of chelated Gd^{3+} typical of lysosomal degradation at very low pH. Thus it is possible that release of profibrotic cytokines precedes the lysosomal degradation of GBCA taken up by receptor-mediated endocytosis. Consequently the formation of tissue retained insoluble Gd^{3+}-species may be secondary or a "footprint" of a receptor mediated cell response (Figure 2). Such a mechanism is entirely consistent with the identification of Gd^{3+}-species localised in areas of dermal inflammation rich in macrophages in NSF (19) and the detection of insoluble Gd^{3+}-species of $<1\mu m$ in diameter confined to areas of fibrosis in NSF tissue (20).

Taken together the above data raise questions about the relevance of dechelated Gadolinium as a trigger in the development of NSF simply because chelated-Gd appears to be capable of stimulating the proinflammatory and profibrotic responses in cells that are able to perform endocytotic functions – fibrocytes, fibroblasts, macrophages and monocytes (Figure 1). It was previously hypothesized that transmetallation, a process in which Gd^{3+} is displaced from the chelate complex, may drive Gd^{3+} accumulation in the tissues and, therefore provide the only

trigger for NSF. However, since it has been shown that direct exposure of human fibroblasts, monocytes and macrophages to GBCA stimulates profibrotic and proinflammatory responses within minutes of exposure (37,40), so it is possible that chelated-Gd^{3+} is not as biologically inert as was previously thought. Such data suggest that Gd-chelate could be a bioactive species associated with NSF in susceptible patients. Such data raise questions about the roles of de-chelated and retained gadolinium as trigger factors in the development of NSF. Clearly such responses do not take place in every patient, thus some form of patient susceptibility is likely. Nevertheless, these data do help explain why cofactors such as high GBCA dose, SIRF and ESRD, dependent oedema and pre-existing inflammation might predispose patients receiving GBCA to develop NSF. In this regard:

High single dose rather than repeated standard doses of GBCA carries the greatest risk *in vivo* (5, 11) accords with the concentrations of GBCA that are required to stimulate inflammatory cells and fibroblasts *in vitro* (36, 37, 38, 39, 40).

The increased exposure of interstitial tissue to GBCA brought about by the extended half life in ESRD and SIRF (2) accords with the effect of GBCA on macrophages and fibroblast inflammatory and fibrotic reactions *in vitro* (36, 37, 38, 39, 40).

The occurrence of NSF in temporal proximity to inflammatory and thrombotic episodes in affected patients (9, 42) suggests that macrophages and inflammatory cells may have been primed by these events and, thus, capable of an enhanced or exaggerated response to noxious agents accords with the observations that monocytes and macrophages can be directly stimulated by GBCA *in vitro* (36, 37).

Dependent oedema / fluid overload leading to enhanced exposure of interstitial tissue to GBCA *in vivo* (6) accords with the observation that fibroblasts can elicit a profibrotic response *in vitro* (38, 39, 40).

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Table 1:

Thermodynamic Stability (K_{therm}) constants of 5 GBCA.

	Dotarem Gadoterate meglumine	**ProHance** Gadoteridol	**MultiHance** Gadobenate dimeglumine	**Magnevist** Gadopentetate dimeglumine	**Omniscan** Gadodiamide
Stability Log (K_{therm})	**25.4**	**22.8**	**22.6**	**22.1**	**16.8**

Table 2:

Conditional Stability (K_{cond}) constants of 5 GBCA.

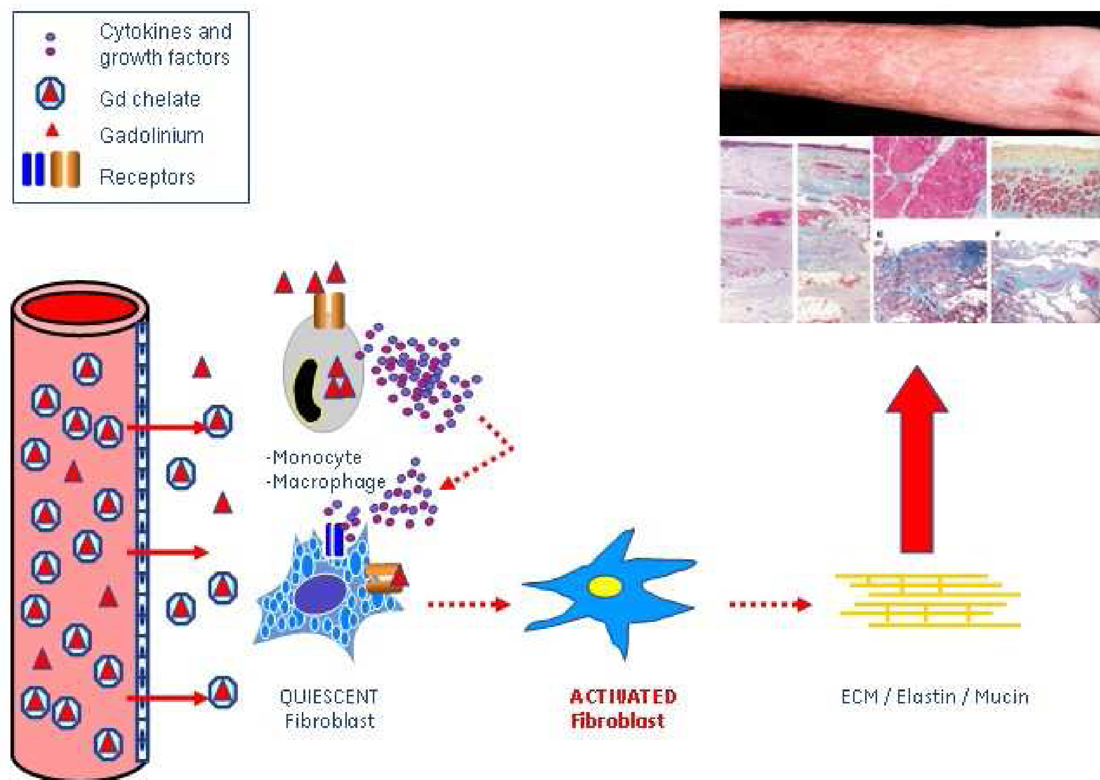
	Dotarem Gadoterate meglumine	**ProHance** Gadoteridol	**MultiHance** Gadobenate dimeglumine	**Magnevist** Gadopentetate dimeglumine	**Omniscan** Gadodiamide
Stability Log (K_{cond})	**19.0**	**17.1**	**18.4**	**17.7**	**14.9**

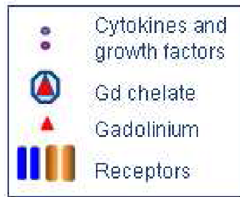
Figure 1:

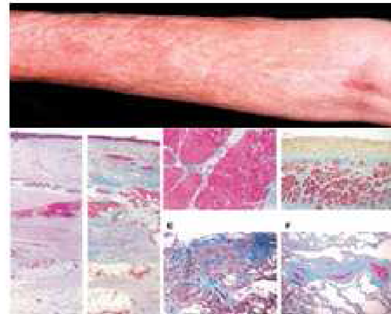
The following schematic of the proposed mechanism shows how the retention of chelated gadolinium, occurring after high dose, ESRD and pre-existing inflammation, might stimulate proinflammatory and profibrotic responses that are consistent with those seen in NSF.

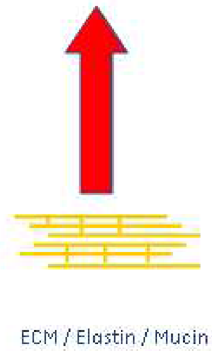
Figure 2:

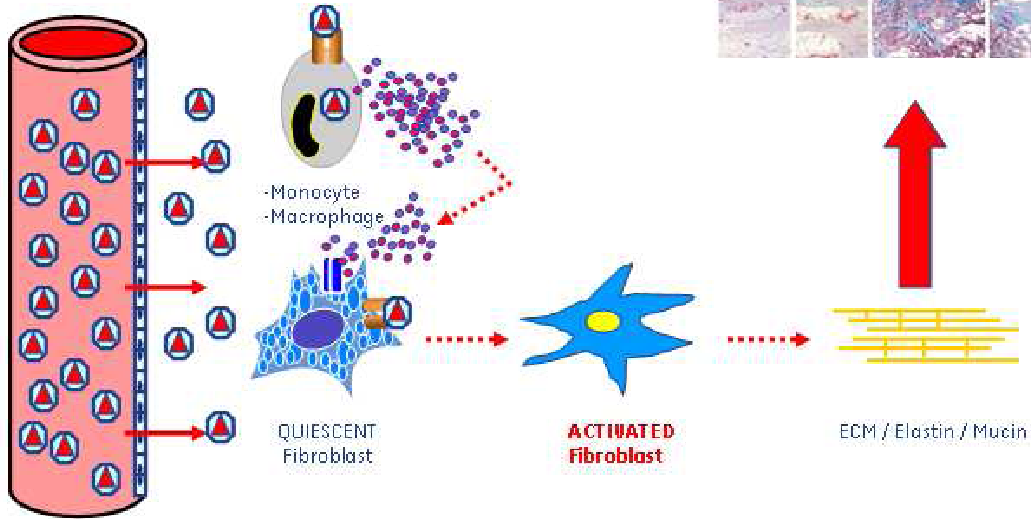
The following schematic suggests that the release of profibrotic cytokines following receptor mediated endocytosis of chelated gadolinium might precede and bring about the formation of tissue retained insoluble Gd^{3+} in NSF.


Cytokines and growth factors
Gd chelate
Gadolinium
Receptors
-Monocyte
-Macrophage
QUIESCENT
Fibroblast
ACTIVATED
Fibroblast
ECM / Elastin / Mucin


Cytokines and growth factors
Gd chelate
Gadolinium
Receptors






-Monocyte
-Macrophage
QUIESCENT Fibroblast
ACTIVATED Fibroblast
ECM / Elastin / Mucin