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$\alpha 6\beta 1$ Integrin and laminin E8: An increasingly complex simple story

SIMON L. GOODMAN

Nephrology Research Laboratory, Medical Clinic IV, University of Erlangen-Nürnberg, Erlangen, Germany

Regulation of interactions between cells and the extracellular matrix lies at the heart of such fundamental biological problems as the generation of pattern during embryogenesis, the process of cancer metastases, organogenesis, and the redistribution of cells during inflammatory responses. In many of these processes, cells come into intimate contact with components of the basement membrane. Basement membranes (BMs) are heterogeneous, highly specialized, 100 to 200 nm thick sheets of matrix interposed between all epithelia, endothelia, skeletal muscle, nerve and fat bodies, and the surrounding stroma. They are distinguished by a characteristic trilamellar morphology in the electron microscope and by a distinct molecular composition. BM functions as filter in the glomerulus, where two basement membranes are directly apposed. During development, and probably during pathological processes, cells also interact with isolated basement membrane components, that is, components not associated into an intact BM.

This brief review will consider what is known about cell interactions with laminin, now known to be a family of BM molecules, and their receptors at the cell surface. In particular, the interactions of cells with what is currently thought of as the major cell binding site in the molecule, in the E8 protease fragment, will be emphasized. There have been several excellent recent in-depth reviews covering laminin [1], and BMs in general [2].

In addition to providing mechanical support, isolated purified BM components have been shown to provoke diverse responses from cells, including cell attachment, growth, differentiation and movement. In addition, they may also bind and locally concentrate growth factors classically thought of as soluble. Thus, a picture is emerging of BMs as solid phase signaling machines, that convey information to cells in their immediate vicinity that bear the necessary surface receptors.

Laminin is a behavioral regulator in the BM

Adult BMs have a composition distinct from the interstitial matrices. The major components are collagen type IV, heparan sulphate proteoglycan, laminin and nidogen. The minor components are numerous (as reflected by bands on SDS gels), but are less well characterized (refer to M. Weber, this issue). The current model of the BM proposes that an extended two-dimensional 'chicken-wire' net of collagen IV molecules is bound by laminin and the proteoglycan. Nidogen binds strongly to laminin, to collagen IV and to heparan sulphate proteoglycan. Laminin binds to itself and to collagen IV and heparan sulphate proteoglycan. Thus the major components are woven together into a cross linked and insoluble network. Immunohistological methods suggest that the orientation and exposure of laminin, and probably of the other BM components, varies from region to region of the BM. One of the best characterized examples of this is in kidney. Such varied exposure presumably reflects differing functions of the different BM regions, but just what these represent in terms of cell-matrix interaction remains to be shown.

At least two of the four major BM components, laminin and collagen IV, may promote cell adhesion when coated on otherwise non-adhesive surfaces. The activities of laminin, in addition to its adhesion-promoting properties, can stimulate a number of other biological responses. These include cell differentiation, proliferation, polarization, chemokinesis and chemotaxis. Current work in the field is focused on delineating the domains of the molecules which provoke these responses, and the receptors at the cell surface which recognize them. It is unlikely that all cells in contact with the BM move, proliferate and differentiate. Faced with such a gamut of potential biological stimuli, the cell must somehow protect itself from inappropriate signals. There appear to be three ways this protection can be effected: absence or inactivation of the necessary laminin receptors at the cell surface; shielding of the ligand sites in laminin; expression of an isoform of laminin lacking the critical ligand sites for which cells in the vicinity bear receptors.

Structure of laminin

Laminin can be purified by mild procedures from the murine Engelbreth-Holm-Swarm (EHS) tumor. Laminin binds calcium ions with moderate affinity, and calcium is apparently involved in its interactions in the BM; divalent cation chelators will extract laminin at neutral pH and physiological salt concentrations from EHS tumors [3]. The resulting molecule extracts in complex with nidogen, and the complex can be disrupted by incubation with 2 M urea. By rotary shadowing electron microscopy, EHS laminin appears cruciform with two globular domains at the ends of each short arm of the cross, and one at the end of the long arm. Isolated in this way, the molecule is a glycosylated heterotrimer, consisting of two closely related B chains, B1 (molecular wt 220,000) and B2 (molecular wt

210,000), and an A chain (molecular wt 440,000). Several murine, human and Drosophila laminin chains have been cloned and sequenced. Structural studies have revealed that individual chains form the short arms then intertwine in a coiled-coil α-helix to descend the long arm to the COOH-terminal of the chains. The long-arm terminal globules are composed of A-chain alone. Laminin isolates from sea urchin, leech, fruit fly, and human have similar appearance under the microscope, following rotary shadowing, but with morphological variations in the short or long arms. A more detailed discussion can be found in a recent review [1].

The protease digestion fragment E8

Many biological activities of laminin disappear on separation of the component chains, in marked contrast to the activities of collagens and fibronectins, where substantial activities may be retained in small peptide fragments. But it has proved possible to study the biological activities of laminin protease fragments. Pepsin, chymotrypsin and elastase digestion of laminin all give rise to a series of discrete fragments which can be purified to homogeneity by standard chromatographic procedures as judged by SDS-PAGE and by electron microscopy.

Early studies concentrated on pepsin and chymotrypsin digests, which tended to destroy the long arm of the molecule, leaving three short arms with or without globular domains, the '1' fragments (P1, C1). These '1' fragments of EHS laminin bear an RGD− sequence (LRGDNG− at residue 1122 to 1127 of the A-chain), which for many (if not all) cells is sterically occluded by an adjacent globular region in the native molecule. Cell attachment studies suggest that it is only exposed following protease digestion [4]. Its relevance in cell recognition of intact laminin is still being investigated. Such 'cryptic' sites may play a role during tissue remodeling where proteases attack laminin and might expose them, thus activating novel surface receptors. Digestion with elastase gave in addition to a '1' fragment (E1) a series of other discrete products, one of which, E8, has several highly interesting activities [5, 6].

E8 is an ≈250 kD fragment comprising the lower 35 nm of the long arm and lacking the two most COOH-terminal globular domains (G4 and G5: the E3 fragment) of the A-chain. The NH2-terminal amino acids of E8 have been identified at residue 1540 on B1, 1329 on B2 and 1887 on A− [7, 8]. E8 promotes neurite outgrowth, stimulates the attachment and spreading of many cells, supports the locomotion of myoblasts and neural crest cells, and is responsible for polarization of kidney tubule epithelia.

At least the major attachment promoting sites in the molecule, in both the intact laminin and the E8 subfragment, are lost when the tertiary structure of the molecule is altered by mild denaturation with urea or heating above about 60°C [6, 7, 9]. This loss of activity coincides with unfolding of the coiled-coil α-helix of domain I in the long arm of the molecule, as observed by circular dichroism [7]. Thus, the E8 cell attachment sites are clearly distinguished from the attachment sites of fibronectin and vitronectin, where an isolated tetrapeptide can support cell attachment, and one role of the surrounding protein appears to be to modify the configuration of this sequence to match specific receptors.

Nevertheless, there have been reports that polypeptide fragments of laminin from domain I have numerous biological activities (that is, in the region of the coiled coil, for example, those containing the sequence IKVAV [10]). It may become easier to reconcile these two streams of evidence when the receptors that interact with IKVAV are characterized. There is convincing evidence that many activities mediated both by intact laminin and by its protease fragments function over integrins. This evidence will be discussed in detail below.

E8 is a stimulator of polarization

The generation of polarity is a basic phenomenon in living organisms. The E8 region of laminin appears to be capable of inducing polarizing activity in some cellular systems. The four systems where the most intense study has occurred are: initial attachment of cells, the outgrowing neurite, the locomoting skeletal muscle myoblast, and the polarizing kidney tubule epithelium.

Initial cell attachment

A cell in suspension is not formally polarized. However, given a suitable stimulus, many cells can attach to a substrate where they rapidly reorganize membrane and cytoskeleton to produce apical and basal surfaces with distinct molecular compositions.

Although the initial cell biology of E8 revealed its ability to polarize attached neurites, it was alone unable to support their attachment. Subsequently we discovered that E8 was also a strong promoter of cell attachment in a wide variety of cell types [9, 11]. Subsequent studies support the view that the usual sites that support laminin-mediated cell adhesion in tissue culture lie within the E8 fragment [12–19]. It is also clear that some cell types have the ability to simultaneously use more than one type of laminin receptor to recognize different regions of the molecule [15, 20, 21]. The reason for this apparent functional redundancy is not yet clear, but may be related to the secondary transmembrane signals that cells receive following attachment to laminin.

Recently, E8 has been successfully digested further with trypsin to produce ≈125 kD subfragments T8 (NH2-terminal residues: B1 1679, B2 1473, A 2009) and T8′(NH2-terminal residues: B1 1679, B2 1473, A 2054) [7]. These fragments have both attachment promoting activity, and depend on the same receptors as E8. Unfortunately, yields of T8 and T8′ are very low (≈0.01% of starting material), but in contrast to both E8 and whole laminin, the truncated B1-B2 and A chains in T8 and T8′ can reassociate following separation in vitro to give biologically active heterotrimer, which opens the way for molecular biological investigation of the cell attachment sites on the molecule (Dr. R. Deutzmann, personal communication).

Interestingly, the T-fragments have revealed a possible interaction between the long arm and its globular terminus, in the generation of cell attachment sites. Fragment T8R, identical to T8 but lacking G2, G3 and much of G1, does not support cell attachment, but neither does the isolated G1-G3 globule. This hints that G1-G3 interacts in some way with the lower regions of the arm. The IKVAV sequence in the A-chain, reported to be especially biologically active in neurite outgrowth, is also located in this region of the long arm [22]. Monoclonal antibodies against laminin that strongly block cell attachment also recognize the region at the juncture of the long arm and the A-chain COOH-terminal globule [19].

Neurite formation: Stimulation by E8

For cells to move anywhere, anterior-posterior polarity must somehow be induced. One of the most obvious examples of this breaking of cellular symmetry is the formation of neurites, long axon-like processes which are elongated by a semi-autonomous motile region, the growth cone, located at the tip of the process. The mechanisms which control the paths taken by outgrowing nerves during development are unknown. As both neural connections and the paths taken by the outgrowing axons are precisely regulated, a seductive hypothesis is that environmental signals may mark the route the axons follow. Indeed, ablation studies have shown that the growth cone senses its immediate environment, before going on to the next environmental 'signpost,' all the while attached by its neurite to the cell body. Laminin may act as one environmental signpost.

NGF can induce neurites in isolated peripheral nerve ganglia in culture. However, for some time laminin has also been known to promote neurite outgrowth from neurones [23]. In contrast, collagen I and fibronectin are only poor inducers. The source of this biological activity is located in E8 [5]. Other fragments of laminin only poorly stimulate neurite outgrowth, thus it is conceivable that during development, outgrowth might be promoted in a given direction by a local environment rich or poor in laminin/E8. T8 but not T8′ have neurite outgrowth-promoting activity (*Initial cell attachment*, above) [7]. This would locate the critical site within the A-chain residues 2009 to 2054, and conflicts with other data suggesting that astrocyte process outgrowth is mediated by the B2-chain [24], or by B1-B2 heterodimers [25], while the PA22-2 peptide (containing IKVAV) has also been shown to have outgrowth promoting activity [22]. Taken together, these data suggest that an interaction between A, B1 and B2-chains generates a structure that promotes outgrowth. The modification of any of the chains in the vicinity destroys the biological activity. In addition to its role in promoting neurite outgrowth, E8 also stimulates attachment, proliferation and differentiation of embryonal neuroepithelial cells [18].

Myoblast locomotion: Stimulation by E8

In common with many embryonal cells, skeletal muscle myoblasts migrate into their developmental target from distant sources (in the dermomyotome of the somite). Once there, they become non-migratory, fuse into multinucleate myotubes and become enveloped in BM. The mechanisms by which such migrations are controlled are of intense interest. Furthermore, following damage to the adult muscle fibers, a population of muscle stem cells, the satellite cells, reiterate many of the events of the embryogenesis, including migration, replication and differentiation, against the BM of the damaged muscle fibre. We therefore studied whether laminin could affect the various processes of muscle development and differentiation.

Not only did laminin promote adhesion of the myoblasts, but it also promoted their polarization to spindle-shaped bipolar morphologies, accelerated their cell cycle, stimulated differentiation into fusion-competent myotubes, and triggered rapid locomotion across the substrate [26–29]. In direct contrast, fibronectin, although an excellent stimulator of adhesion, failed to stimulate proliferation and differentiation, and provoked neither bilateral polarization nor locomotion. Similar laminin stimulations of locomotion were obtained by others using a rhabdomyosarcoma cell line [13], and in avian systems using primary cultures of neural crest cells [12]. In all three systems the E8 region of laminin has been described as the active domain, while fragments from the three short arms have no locomotion stimulating activity [12, 13, 29]. As there is no direction in the systems studied, laminin is here acting as a chemokinetic stimulus. This contrasts with the chemotactic activity described for the YIGSR-peptide in the B1-chain [30].

One interesting aspect of the stimulation was that it was strongly dependent on the quantities of laminin in the substrate [12, 29]. There is an obvious necessity that in order for traction to be generated, enough laminin has to be present to allow cells to stick. It was a surprise to find that as the concentration of laminin was increased, the locomotion of myoblasts decreased sharply. This was possibly due to the substrate becoming too adhesive. On the other hand, irrespective of the stickiness of the fibronectin substrate, as judged by cell attachment assay and morphology, there was no significant stimulation of locomotion. There was a close overlap between the stimulatory effect of laminin and of its E8 subfragment, suggesting that essentially all the locomotion stimulating activity resided in E8 [29].

It is particularly interesting that all the processes of muscle repair, in several aspects reiterations of processes last seen during development, are stimulated by the E8 fragment of laminin. It is therefore possible that exposure of BM following muscle damage may participate in the activation of quiescent satellite cells, and that during embryogenesis, the location of laminin-rich environments or the up regulation of E8 receptors influences skeletal muscle development [31].

Epithelial polarization in kidney: Stimulation by E8

Kidney is a tissue constructed during development from invading and local cell populations, and whose development involves a series of epithelial-mesenchymal and mesenchymal-epithelial transitions. In vivo, loose mesenchyme is induced to condense about the ingrowing ureteric bud, and polarizes to form the kidney tubules. In vitro, many tissues will induce this condensation of epithelial tubules, even when separated from the mesenchyme by a microporous filter, and this provides one of the best models for investigating how the polarization is induced.

Antibodies against the E8 and E3 fragments of laminin will block the induction of epithelial polarization in isolated kidney mesenchyme. Antibodies directed against other regions of laminin did not affect the polarization. Elegant studies in organ culture have shown that there is a precise spatial and temporal relationship between the expression of laminin A-chain and the induction of polarization. B1- and B2-chains, meanwhile, were constitutively and continuously expressed in the system [32]. The specificity of these blocking activities argues strongly for a direct effect of laminin in the system, and this was further supported by the kinetic of synthesis of the A, B1 and B2 chains. As mentioned above, intact laminin heterotrimer are required to promote most forms of cell attachment over the E8 region in vitro. The involvement of E8 was confirmed by receptor studies.

Cellular receptors for the E8 fragment

Combinations of affinity chromatography, antibody blocking, receptor expression studies, immunolocalization and transfection studies have given us the following picture of the cell surface receptors for laminin. In systems that resemble the extracellular milieu in vivo, that is, with divalent cations magnesium and calcium at low millimolar concentrations, initial cell attachment to laminin usually appears to be mediated by members of the integrin superfamily of receptors [reviewed in 33, 34]. Nevertheless, it should be noted that there is a recurrent blooming of non-integrin laminin binding proteins with a molecular weight of 68 kD, which have been proposed to be laminin receptors [30, 35]. These molecules lack classical transmembrane sequences, can be eluted from laminin columns with the YIGSR sequence derived from domain III of the B1-chain, and this peptide can also affect diverse cell behavior. As these molecules have not yet been reported to interact with E8 they will not be considered further here.

Intact EHS-laminin will bind in a divalent-cation dependent manner to five β1 series integrins [33, 34], depending on which cell type is investigated; α1β1, α2β1, α3β1, α6β1 and α′β1. Of these, α1β1 clearly binds in the E1 region, and in some cell types, the site is available in the native molecule as well as the protease digestion fragments [21, 36]. The binding site in laminin of α2β1 is not yet characterized [37].

α3β1 binds within E8 near the COOH-terminal globule [38, 39], as do α6β1 and α′β1 [14, 40, 41]. The function of α3β1 is far from clear. It can localize in talin-positive focal adhesions on laminin, collagen I and fibronectin, but antibodies directed against the molecule have little or no effect on attachment. The best proposal at present is that it is a subsidiary molecule that supports 'major integrin-mediated' adhesions [42]. In view of its curious distribution, this is not an altogether satisfactory explanation. A study of the dynamics of the adhesive structures containing α3β1 as opposed to other integrins may throw some more light on this problem.

α6β1 is a major laminin receptor [43]; indeed, antibody blocking studies suggest that it is the sole laminin receptor in many cell types. It binds, as does α3β1, toward the COOH-terminal of the E8 rod [14, 40, 41]. However, in distinct contrast to antibodies to α3, which block attachment poorly, antibodies against the α6 chain are intense blockers of cell attachment to laminin and to E8 (but not to other matrix molecules). α6β1 can be immunoprecipitated from the surface of cells whose attachment is blocked by the antibody, and in such cells α6β1 localizes in talin-positive focal adhesions on laminin substrates (H. Grenz and SLG, *unpublished observations*).

In the polarizing kidney epithelia (described above), it has been convincingly demonstrated that cell interactions employing α6β1 integrin induce polarization of the tubule epithelial cells; antibodies to α6 strongly disrupt the development of the epithelium [44]. In three cell types, human melanoma [45, 46], murine skeletal muscle satellite cells and the Rugli rat glioblastoma line, α6β1 is at best weakly expressed, antibodies against α6 have no effect on cell attachment, and attachment to E8 appears to be mediated by a further β1 series integrin [41]. To avoid numerological conflicts, this is currently termed α7β1 for the melanoma and α′β1 for the muscle and Rugli cells. They are homologous molecules. As α′β1 is the only receptor for E8 that can be identified on myoblast surfaces it seems possible that it is the myoblast 'locomotion receptor' whose interaction with ligand activates the motile machinery of the cells (*Myoblast locomotion*, above). We are in the process of cloning α′. α′ is a previously unsequenced α-chain, and is most homologous to α6. α′β1 complexes can be isolated both from myoblasts and Rugli cells on both laminin and E8-affinity columns [41]. The reasons for multiple E8 receptors are not entirely clear, but it is interesting that cells which synthesise α′ locomote on the E8 fragment of laminin. How far this correlation holds awaits much further study.

In vivo, unilaterally injected hybridoma cells producing antibodies against the β1 integrin chain inhibit the migration of avian myoblasts from the dermomyotome into the periphery [47]. Given the manifold roles of the integrins, this may be no surprise, but serves to comfort the tissue culturists in the field. It is not yet clear which α-chain is being employed in this system in vivo. Unfortunately, the defining anti-α6 antibody, GOH3, reacts poorly in avian systems.

A maelstrom of laminins?

What is the function of so many receptors for laminin? Perhaps the expression of various combinations of receptors leads to the induction of specific patterns of secondary intracellular signals from a homogeneous environment. This in turn results in defined cellular behavior, and may include specific activation or inactivation of genes. Another possibility is that in the normal tissues the laminin environment is not uniform, that the EHS tumor laminin is anomalously capable of interacting with laminin receptors that laminin from normal tissues does not activate. Thus, it is interesting to find that native tissue laminin is neither uniform, nor is it the same as that found in the EHS tumor, nor do cells respond to it as they do to the EHS laminin, nor do they use the same receptors to do so as they do for tumor laminin.

Several variant 'isoforms' of laminin have been identified by cDNA cloning, or identified using anti-BM monoclonal antibodies. Schwannoma cell line RN22 secretes a B1-B2 chain dimer, deficient or lacking in A-chains [25]. A B1-chain homologue (associated with unknown A-chains: 'S-laminin' [48]) has been reported to stimulate the attachment of ciliary ganglion, but not central and sensory neurones; conventional laminin stimulates the attachment of all three forms [49]. An A-chain homologue, M-laminin [50], or merosin has considerable homology to the COOH-terminal of the EHS-laminin A-chain, and seems to form an M-B1-B2 complex in human placenta [51] while an A′ chain of some 250 kD has been reported as an A′-B1-B2 complex in endothelia [52]. The biological activities of these isoforms has yet to be well characterized.

Basement membranes are uniformly rich in laminin when visualized with polyclonal antibodies against murine EHS-laminin (characteristically reactive with B1-, B2- and A-chains). However, A-chain and isoform-specific antibodies tell different stories. For example, S-laminin is found in the muscle BM exclusively at neuromuscular junctions, and in the kidney, within the glomerulus [48, 53]. The cell surface receptors for S-laminin within the glomerulus, if any, have not yet been identified. Presumably, such restricted localization reflects a specific function. A general finding is that in adult organisms,

B-chains are dominant and A-chains are weakly or not expressed. In one of the few systems where it has been rigorously studied, during murine kidney development, A-chain synthesis is rigorously controlled. In the endothelia it is also apparent that the secretion of laminin trimers and their assembly is a very complex process [52]. As in most cell biological studies isolated B-chains are inactive, the isoforms of laminin may be the molecules more relevant to non-tumor biology. It seems likely that it is the laminin isoforms and the cell surface receptors that perceive them that will increasingly become a focus of research, eclipsing EHS-laminin. Indeed, the significance of structural variations between EHS- and other isoforms of laminin can already be probed by using anti-receptor antibodies.

Although protease digested human placental laminin has been available for some time [54], intact molecules from species other than mouse, and sources other than BM tumor, have not been available in quantities sufficient for cell biology until recently. In a study that marked a watershed in the field, a simple and rapid purification of tumor laminin was devised, and used to isolate for the first time an intact normal laminin (from murine heart) [3, 55]. The chelator extraction method allows milligram quantities of intact placental laminin to be isolated. This isolate contains more than one isoform (J. Brown and R. Timpl, *personal communication*, and SLG, *unpublished observations*). Intact placental laminin stimulates cell attachment of rat, human and mouse cells, with similar concentration dependency and kinetic to EHS-laminin [56]. And although the microscopic form of human placental laminin is similar to the EHS-molecule, some biological activities are not. Not only is the coiled-coil alpha helix in the long arm considerably more thermally stable than in EHS-laminin, but also it is apparently not recognized by the same receptors. Initial studies indicate that α6β1, for example, is not the major cell surface receptor for human laminin, either on murine or human cells, both of which use only α6β1 to attach to mouse EHS-laminin. Neither α2β1, α3β1, nor α6β1 appear to be involved in interaction with the native human protein [56], and, in the absence of other receptors, this points in the direction of the αV series integrins. These are highly sensitive to RGD-peptides, so the mechanism of attachment can be readily examined.

It remains to be seen how many isoforms of laminin exist in vivo, what are their various receptor specificities, and what biological events they trigger. It is unfortunate that the major cell attachment sites in both the EHS and the human placental (Hp?) isoforms both require tertiary structure, as this suggests that molecular biological approaches to the problem by expressing and manipulating the chains to identify functional sites may be difficult.

Conclusion

In summary, EHS-laminin supports many biological activities and does so primarily by binding integrins at the cell surface. Limited in vivo data supports the view that laminin-integrin interaction can modulate cell behavior. The major region of the molecule that promotes initial cell interactions is located at the COOH-terminal of the long arm, within the E8 fragment, and the most specific receptor for this site appears to be the α6β1 integrin—a primary laminin receptor on most cells examined so far.

However, evidence is emerging that the EHS model may not reflect the state of affairs throughout native BMs, and the laminin isoforms isolated from human placenta can be shown to have structure and function different from those of the EHS model. Taken together with immunohistological studies, there seems to be considerable heterogeneity in the laminin composition of the BMs, and this in turn has diverse effects on cells that interact with them.

At the molecular level, the events that E8 triggers in cells are extremely complex. For example, cell migration requires the repeated attachment and detachment of specified regions of the cell surface to the substrate, the generation of directed force, and the organized translocation of the cell contents in the correct direction. Each of these activities is exquisitely tightly regulated and requires the coordination of numerous subsystems within the cell. Similarly, massive intracellular reorganization accompanies epithelial polarization.

This is, perhaps, the great fascination of the E8 fragment, to discover how it sets such complex trains of events in motion. We can look forward to an immediate future in which the nature of the secondary messages that accompany laminin receptor-ligand interactions will be sought and molecular biological methods brought to bear on the ligands and their receptors to define with great precision the molecular mechanisms of the interaction.

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Reprint requests to Simon L. Goodman, D.Phil. Nephrology Research Laboratory, Medical Clinic IV, University of Erlangen-Nürnberg, Loschgestr. 8-½, 8520 Erlangen, Germany.

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