Short Communication

Association of Active Extracellular Signal-Regulated Protein Kinase with Paired Helical Filaments of Inclusion-Body Myositis Muscle Suggests Its Role in Inclusion-Body Myositis Tau Phosphorylation

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The possible role of extracellular signal-regulated kinase (ERK) in the pathogenesis of inclusion-body myositis (IBM) was investigated by immunostaining the active phosphorylated form of ERK in muscle biopsies of six IBM and 14 control patients. Between 80% and 90% of IBM vacuolated muscle fibers contained well-defined ERK-immunoreactive inclusions, which were co-localized by light microscopy, with phosphorylated tau in 70 to 80% of those fibers. Immunoelectronmicroscopy colocalized ERK to small amorphous tufts adjacent to the muscle fiber paired-helical filaments. Strong ERK immunoreactivity was also present at the postsynaptic domain of all human neuromuscular junctions. Our study suggests 1) that ERK, a signal transducer, might play a role in IBM pathogenesis, including participation in the pathological phosphorylation of IBM tau; and 2) that signal transduction abnormalities may be a component of the IBM pathogenic cascade. Our novel immunolocalization of ERK at the postsynaptic domain of human neuromuscular junctions supports a role in transcription of junctional-protein genes. The ERK localized in nonjunctional regions of IBM fibers may underlie the known pathological up-regulation of junctional proteins there. (*Am J Pathol* 2000, 156:1835–1840)

Sporadic inclusion-body myositis (IBM) is the most common, debilitating, and progressive muscle disease of patients 55 years and older.¹ The characteristic features of IBM muscle pathology are: 1) various degrees of mononuclear-cell inflammation, and 2) vacuolar degen-

eration of muscle fibers, accompanied by intrafiber amyloid β (A β) deposits and focal cytoplasmic accumulations of 15- to 21-nm-diameter, paired-helical filaments (PHFs) containing phosphorylated tau, both of which are congophilic.^{2,3} The abnormalities within IBM muscle fibers have many similarities to those in the brain of Alzheimer's disease (AD) patients. In addition to congophilia, tau-containing PHFs, and accumulations of A β , they include accumulations of two other epitopes of A β -precursor protein (A β PP),⁴ A β PP mRNA,⁵ presenilin-1,⁶ and several other "Alzheimer characteristic" proteins.¹ Remaining unknown in both IBM and AD are the factors leading to the abnormal accumulation of various proteins and the molecular pathways responsible for PHF formation in the respective tissues. Oxidative stress, in association with aging, has recently been postulated to be an important component of IBM and AD pathogenesis.^{1,7}

In AD neurons, active extracellular signal-regulated protein kinase (ERK) has been immunocolocalized with both phosphorylated tau^{8,9} and 8-hydroxyguanosine,⁹ a marker of oxidative damage. Accordingly, ERK was proposed to link oxidative stress to the AD brain pathology.⁹ In our exploration of the pathogenic cascade leading to IBM, including mechanisms of tau phosphorylation, and the possible influence of oxidative stress, we studied the expression of the active, phosphorylated ERK in IBM muscle biopsies. ERK is a serine/threonine protein kinase that was shown in cell culture to be activated by reactive oxygen species.^{10,11} *In vitro*, ERK phosphorylates the same residues of tau that are phosphorylated in AD neurofibrillary tangles.^{12,13}

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Materials and Methods

Patients

Immunocytochemical studies were performed on sections of diagnostic muscle biopsies obtained from 20 patients with these diagnoses: IBM ($n = 6$), dermatomyositis ($n = 2$), polymyositis ($n = 2$), morphologically non-specific myopathy ($n = 2$), amyotrophic lateral sclerosis ($n = 3$), chronic peripheral neuropathy ($n = 1$), non-IBM vacuolar myopathy ($n = 1$), and normal muscle ($n = 3$). Diagnoses were based on clinical and laboratory investigations, including 18-reaction histochemistry of the muscle biopsy.

Light Microscopy Immunocytochemistry

Immunocytochemistry was performed on 10- μ m-thick sections of the fresh-frozen biopsies. The sections were either unfixed or prefixed with acetone or 2% paraformaldehyde. Peroxidase-antiperoxidase and immunofluorescence procedures were performed as described.^{2-4,6} To localize active ERK we used two rabbit polyclonal antibodies (New England Biolabs, Beverly, MA; and Promega, Madison, WI), diluted 1:20 to 1:100. Both antibodies specifically recognize dually phosphorylated epitopes, p-Ser202/p-Tyr204 and p-Ser183/p-Tyr185, on ERK1 and ERK2, respectively (8, 9 and immunoblots provided by the companies). Double-immunofluorescence used rabbit polyclonal antibody against ERK combined with: 1) mouse monoclonal antibody AT8 (Innogenetics, Alharetto, GA), a well-characterized specific marker of PHF tau in both AD and IBM that recognizes dually phosphorylated Ser202/Thr205 epitope (according to the sequence of the longest human tau isoform);^{3,14} 2) a mouse monoclonal antibody against desmin (Chemicon International, Inc., Temecula, CA), which identifies regenerating (positive) and necrotic (negative) muscle fibers; 3) α -bungarotoxin (α -BT) conjugated to fluorescent marker Alexa (Molecular Probes, Eugene, OR), which binds to the postsynaptic nicotinic acetylcholine receptors at neuromuscular junctions (NMJs); 4) a mouse monoclonal antibody against CD163 (clone Ber-MAC3; DAKO, Carpinteria, CA), thereby recognizing human macrophages; or 5) Hoechst 33342 (Molecular Probes), a DNA-binding dye illuminating nuclei.

To block nonspecific binding of antibody to Fc receptors, the sections were preincubated with normal goat serum diluted 1:10. Omission of the primary antibody or its replacement with nonimmune serum or irrelevant antibodies were used to control for specificity, as described.^{2-4,6}

Immunoelectronmicroscopy

This was performed on 10- μ m, unfixed frozen sections adhered to the bottom of 35-mm Petri dishes.^{2-4,6} Active ERK was immunolocalized with a HRP-conjugated secondary antibody, or was doubly-immunolocalized with monoclonal SMI-31 antibody (Sternberger Monoclonals,

Figure 1. Light microscopic immunolocalization of ERK using peroxidase-antiperoxidase reaction (A–C). Abnormal muscle fibers of IBM have strongly ERK-immunoreactive squiggly, linear, or dotted inclusions of various sizes. **D** and **E**: Dermatomyositis; on serial sections, the same cluster of macrophages invading a muscle fiber, and a few individual macrophages (arrows), are darkly immunostained with a macrophage marker (**D**) and with anti-ERK antibody (**E**). Original magnification, $\times 600$.

Baltimore, MD), using two appropriate secondary antibodies conjugated either to 10-nm or 5-nm gold particles.^{2-4,6} SMI-31 antibody cross-reacts with PHF- phosphorylated tau of both IBM muscle³ and AD brain,¹⁵⁻¹⁷ and it recognizes the dually phosphorylated epitope Ser 396/Ser 404.¹⁷

Results

Light Microscopy Immunocytochemistry

IBM

In all IBM biopsies, 80 to 90% of the vacuolated muscle fibers and 10 to 15% of nonvacuolated ones had strong ERK immunoreactivity (IR). ERK-IR was in the form of numerous, well-defined squiggly, linear, or dotted inclusions (Figure 1, A–C, and Figure 2, A and C). Occasional muscle fibers, in addition to the immunoreactive inclusions, had a slight diffuse cytoplasmic ERK-IR. These patterns of immunostaining were present in both unfixed and fixed sections. In unfixed or acetone-fixed sections, ERK-IR in the myonuclei was very weak. Paraformaldehyde fixation significantly increased ERK-IR of the myonuclei in all IBM and control biopsies. In IBM, paraformaldehyde combined with Hoechst counterstaining revealed that only a minority of the ERK-immunoreactive inclusions in abnormal muscle fibers were associated with nuclei (Figure 2, F–I, arrows), most being in the cytoplasm.

In 70 to 80% of muscle fibers containing ERK-immunoreactive inclusions, there was close co-localization of them with AT8-immunoreactive inclusions (indicating

Fig. 2. ERK immunoreactivity in sporadic inclusion-body myositis muscle. **A** and **C** closely co-localize with AT8-immunoreactive inclusions (**B**, **D**, and **E**, double exposure), the latter indicating phosphorylated tau on IBM-PHF. In some muscle fibers (lower fiber in **C**), ERK immunoreactivity is much stronger than that of AT8 (lower fiber in **D**). **F–I**: Co-localization of ERK with the Hoechst nuclear marker. Only a minority of the ERK-immunoreactive inclusions are associated with nuclei. Original magnification, all $\times 1,200$. **J–N**: Two normal human NMJs. There is a co-localization of ERK (**J–M**) with α -bungarotoxin (α -BT) bound to the postsynaptic acetylcholine receptors (**K** and **N**). **J** and **K**: ERK and α -BT are closely overlapping as indicated by the yellow fluorescence in **L**. Original magnification, $\times 600$.

phosphorylated tau on PHFs; Figure 2, A–E). However, in some muscle fibers, ERK-IR was either much more abundant than AT8-IR (Figure 2, C–E, lower fiber), or there was no detectable AT8 immunoreactivity.

When primary antibody was omitted or replaced with an irrelevant antibody the above-described immunoreactions were not detectable.

Control Muscle Biopsies

None of the control biopsies contained ERK-immunoreactive inclusions characteristic of the IBM abnormal muscle fibers.

At the postsynaptic domain of the neuromuscular junctions in all control and IBM biopsies that contained them, there was strong ERK-IR. Depending on the plane of section, ERK-IR either strictly overlapped with the bound α -BT or occupied a slightly larger territory (Figure 2, J–N). In all myopathies, macrophages invading necrotic muscle fibers had increased ERK-IR (Figure 1, D and E). Occasionally, weak diffuse immunoreactivity was present in the cytoplasm of necrotic, desmin-negative muscle fibers, independently from ERK-immunoreactive macrophages. The cytoplasm of regenerating fibers was negative, but after paraformaldehyde fixation slightly increased ERK-IR was occasionally present in their nuclei.

Figure 3. Immunoelectronmicroscopy of ERK in IBM vacuolated muscle fibers. **A:** Lower-power electronmicrograph of peroxidase reaction demonstrates the dark reaction product (**top, left**) covering PHFs exclusively and entirely, whereas the adjacent portion of the myofiber (**bottom, right**) is not immunostained (original magnification, $\times 26,400$). **B:** Higher-power double-labeled gold immunoelectronmicroscopy illustrating co-localization of ERK (5-nm gold particles) and AT8 (10-nm gold particles). AT8 is present on the PHFs, whereas most of the ERK immunoreactivity is present on very small tufts of amorphous material adjacent to, and often touching, the PHFs but not directly aligned on them (original magnification, $\times 67,200$).

Immunoelectronmicroscopy

In IBM abnormal muscle fibers, ERK immunolocalized to the clusters of PHFs. When HRP-labeled secondary antibody was used, the HRP reaction product labeled PHFs entirely (Figure 3A). However, gold immunolabeling revealed that ERK was very closely associated with the PHFs but its immunoreactivity was present on very small tufts of amorphous material adjacent to, and sometimes apparently attached to, the PHFs (Figure 3B, arrows). Therefore, it appeared that ERK was not a component of the PHF backbone itself.

Discussion

ERK is a member of a family of mitogen-activated protein kinases (MAPKs), which are serine/threonine kinases involved in the intracellular transduction of signals regulating cell proliferation, growth, differentiation, survival, and death.^{18,19} Two isoforms of ERK, ERK1 and ERK2, have $\sim 90\%$ structural homology and seem to have the same functions.^{18,19} ERK can be activated by many different types of receptors, including receptors for growth factors

(eg, receptor tyrosine kinases such as EGFR and PDGFR). Ligand-binding to a growth-factor receptor initiates a sequence of signaling events involving the Ras pathway and kinases Raf and MEK, which lead to phosphorylation activation of ERK.^{18,19} Other receptors, such as cytokine receptors, G-protein-coupled receptors and integrins, initially use somewhat different signaling steps, but subsequently their signals converge on the cascade initiated by Ras. ERK is activated by phosphorylation of its specific threonine and tyrosine residues. Active ERK, which is the form immunolocalized in the present study, phosphorylates cytosolic enzymes and cytoskeletal proteins, including tau, and also translocates to the nucleus, where it phosphorylates transcription factors that influence expression of various genes.^{18,19}

In a various cell types, ERK was shown to be activated by oxidative stress. Reactive oxygen species are thought to activate ERK through modification of the physiological ERK-activation pathways.¹⁰ For example, Ras, a physiological ERK inducer, can be activated by nitric oxide through nitrotyrosylation of one of its cysteine residues.²⁰ Although activation of ERK protects various cells in culture from oxidant-mediated death,¹⁰ in other cells ERK promotes death induced by reactive oxygen species.¹¹

In the present study, we demonstrated accumulation of active ERK in the IBM abnormal muscle fibers, in activated muscle macrophages, and at the postsynaptic domain of normal human neuromuscular junctions.

The anti-ERK antibodies used in our studies specifically recognize the double-phosphorylated, active form of ERK. In the IBM muscle fibers, at the light-microscopic level ERK-immunoreactive cytoplasmic inclusions were co-labeled with antibodies against phosphorylated tau-containing PHFs. Our immunoelectronmicroscopy with the peroxidase technique demonstrated a high concentration of ERK in the large clusters of PHFs, but the gold technique showed ERK to be in small amorphous tufts among, and often touching, PHFs, but not directly aligned on the PHFs themselves. That appearance suggested that ERK may be associated with formation of nascent PHFs in the tufts, which would be harmonious with the known ability of ERK to phosphorylate tau, which is considered critical to the transformation of tau into PHFs.^{12,13} The phosphoepitopes of tau phosphorylated by ERK *in vitro* are the same ones that are recognized by the AT8 and SMI-31 antibodies^{12,13} used by us to co-localize phosphorylated tau (on PHFs) with ERK, by light microscopy and gold immunoelectronmicroscopy. In some IBM fibers, ERK-IR was either more abundant than that of phosphorylated tau, or there was no detectable tau-IR, suggesting that over-expression of ERK may precede tau phosphorylation. Therefore, our results suggest that in IBM muscle ERK may participate in the pathological phosphorylation of PHF tau. Accordingly, activation of ERK in IBM may be at least one of the underlying causes of PHF formation.

The ERK-immunoreactive inclusions in IBM vacuolated muscle fibers do not reflect macrophages, because our previous study²¹ using double-labeling with a macrophage-specific marker, and the present study using the macrophage marker anti-CD163, demonstrated that vir-

tually none of the IBM vacuolated muscle fibers contained invading macrophages.

Visualization of the nuclear ERK-IR in this study required paraformaldehyde fixation. Possibly paraformaldehyde protected the active ERK epitope from the degradation by endogenous proteases and/or phosphatases, prevented the extraction of ERK from nuclei, or by cross-linking changed the conformation of active ERK to make it more immunoreactive. Since paraformaldehyde fixation was not required to achieve the very strong staining of IBM-PHF, ERK associated with the PHFs may be less vulnerable to degradation/extraction, or much more abundant and thus its detection less influenced by such diminution. The presence of the active ERK in muscle nuclei is consistent with the role of ERK in the regulation of gene expression shown in other cell types.^{18,19} For example, under conditions of oxidative stress ERK, possibly in conjunction with other factors, induces transcription of *c-fos* and *c-jun* genes.¹⁰ ERK also increases phosphorylation and thereby activation of c-Fos and c-Jun proteins,^{18,19} which are components of the AP-1 transcription factor complex that induces expression of the *AβPP* gene.²² Because IBM abnormal muscle fibers have strongly increased *AβPP* mRNA,⁵ and increased expression of c-Jun and c-Fos proteins and Jun kinase (Broccolini and Askanas, unpublished observations, 1999), their increased ERK activity might participate in the *AβPP* gene induction and the c-Fos/c-Jun expression. *Aβ*, an amyloidogenic fragment of *AβPP* that is also increased in IBM muscle fibers,⁴ itself can induce ERK activity in cultured cells.^{23,24} We therefore propose that in IBM increased *AβPP* and *Aβ* lead to oxidative stress which subsequently induces active ERK; and as a result of its activation, ERK stimulates AP-1 transcription factor causing even more of *AβPP* to be produced, creating a self-perpetuating destructive mechanism. Therefore, in IBM muscle fibers, ERK activation may have at least two pathogenic actions, increasing both the phosphorylation of tau and the *AβPP* gene expression.

Our novel localization of active ERK postsynaptically at all studied human NMJs and its virtually exact co-localization with α -BT binding suggest that active ERK may participate in signal transduction of nerve-muscle interaction. Our finding is harmonious with a previous study of cultured animal muscle in which ERK was shown to mediate transduction of a signal from the muscle plasmalemmal neuregulin receptor to muscle nuclei that induces ACh receptor gene activation.²⁵

Our immunolocalizations of active ERK, both at the human normal NMJs and in IBM muscle fibers, are consistent with our hypothesis of a pathological junctionalization of nonjunctional regions of IBM muscle fibers.¹ In the normal innervated mature muscle fibers, genes of the various junctional proteins (proteins accumulated in normal fibers only at postsynaptic junctional regions) are down-regulated in the nonjunctional nuclei located in the vast nonjunctional regions of the muscle fiber. However, in the nonjunctional regions of IBM muscle fibers, many of the junctional proteins, and the mRNAs of some of them, are pathologically accumulated.¹ Accordingly, we hypothesized that those abnormal nonjunctional accumula-

tions in IBM muscle fibers result from a pathological extrajunctional activation of a factor or factors responsible for the normally junction-specific gene expression. Active ERK seems to be a factor mediating junction-specific gene expression,²⁵ and it is prominently present at the normal human NMJ (this study). Its prominent accumulation in nonjunctional regions of IBM abnormal muscle fibers (perhaps because of the oxidative stress, an occult virus, or other factors) suggests that its cytoplasmic activation there may participate in derepressing nonjunctional nuclei and subsequent topographically aberrant production of various junctional proteins.

Conclusion

Our studies demonstrating in sporadic IBM muscle co-localization of ERK with phosphorylated tau at the PHFs suggest a pathogenic role of ERK in IBM tau phosphorylation. Prominent localization of active ERK at the postsynaptic domain of normal human NMJs and in nonjunctional regions of IBM abnormal muscle fibers supports a putative role of ERK in the up-regulation of junctional proteins in both regions, normally and pathologically, respectively.

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