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Enhanced proteolytic activities in cultured fibroblasts of Alzheimer patients are revealed by peculiar transketolase alterations / Francesco Paoletti; Alessandra Mocali. - In: JOURNAL OF THE NEUROLOGICAL SCIENCES. - ISSN 0022-510X. - STAMPA. - 105:(1991), pp. 211-216.

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04 May 2026

Journal of the Neurological Sciences, 105 (1991) 211–216
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JNS 03618

Enhanced proteolytic activities in cultured fibroblasts of Alzheimer patients are revealed by peculiar transketolase alterations

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(Received 8 February, 1991)
(Revised, received 1 May, 1991)
(Accepted 24 May, 1991)

Key words: Transketolase; Alzheimer's disease; Human fibroblasts; Cell cultures; Cysteine-proteases; Limited proteolysis; Protease inhibitors; Isoelectric focusing

Summary

Characteristic alterations of transketolase (TK) in extracts from cultured Alzheimer fibroblasts have previously been reported (Paoletti et al. (1990) Biochem. Biophys. Res. Commun., 172: 396–401). These abnormalities, encountered in 9 out of 13 Alzheimer patients, were revealed following isoelectric focusing and consisted of enzyme forms having unusually high alkaline pI values (alkaline bands). The present work has shown that immunologically detected alkaline bands were progressively expressed when Alzheimer fibroblasts were incubated for three weeks without medium changes. Full expression of the altered enzyme pattern was not linked to relative cell density in the petri dish; rather, it appeared to be dependent directly on the time elapsed since cell confluence was reached. Alkaline bands could artificially be induced also in both crude and pure TK preparations from normal cells by a treatment with commercial proteases, particularly chymotrypsin. Moreover, specific inhibitors of endogenous cysteine-proteases were capable of abolishing TK alkaline bands in Alzheimer fibroblasts thus turning a pathological into a normal enzyme pattern. Results obtained suggest that Alzheimer fibroblasts contain enhanced Ca^{2+}-independent cysteine-proteolytic activities as compared to normal and other pathological cells. These enzymes, exhibiting chymotrypsin-like activity, might exert their degradative effects at the time of cell extraction using TK and probably other cell components as potential substrates. However, peculiar TK abnormalities represent so far an useful biochemical marker detectable in fibroblasts of living Alzheimer patients and closely associated to this neurological disorder.

Introduction

Transketolase (TK; EC 2.2.1.1) is a thiamine pyrophosphate-dependent enzyme and catalyzes two steps of the nonoxidative branch of the pentose-phosphate cycle (Horecker and Smyrniotis 1953; Horecker et al. 1954). Induced thiamine deficiency is known to depress enzyme activity in both human (Kuriyama et al. 1980) and experimental animal tissues (Gigùere and Butterworth 1987) as well as in cell culture systems (Beliveau and Freedland 1980). Therefore the TK assay has been employed as a valuable test for B_1-hypovitaminosis (Brin 1966; Londsale and Shamberger 1980) due to an inadequate dietary intake or malabsorption. Moreover, TK alterations of either kinetic constants for thiamine pyrophosphate or isoenzyme profile have been reported in erythrocytes and brains of patients suffering from neurological impairments as encountered in chronic alcoholism (Mukherjiee et al. 1987), Wernicke–Korsakoff syndrome (Blass and Gibson 1977; Nixon et al. 1984) and Alzheimer's disease (AD) (Sheu et al. 1988).

Previous work from our laboratory (Paoletti et al. 1990) has shown that peculiar TK abnormalities occurred frequently in extracts of cultured fibroblasts from Alzheimer patients, as compared to fibroblasts derived from healthy subjects and patients with other neurological disorders. TK alterations (so-called alkaline bands) were detected following the isoelectrophoretic analysis of cell extracts and specific immunostaining with anti-TK IgG preparations. Altered TK profiles in AD fibroblasts were not due to intrinsic enzyme lability. Rather, alkaline bands were tentatively

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explained as the result of TK limited proteolysis and proposed as a potential biochemical marker of the disease.

The aim of this work was to investigate the expression of TK alkaline bands during cell growth in culture and the mechanisms which might be responsible for their generation in AD fibroblasts. We have been able to show that TK alterations were particularly evident in resting cultures within 1–2 weeks following the attainment of confluence. Moreover, TK alkaline bands comparable to those of AD cell extracts could be obtained by treating either crude or pure normal enzyme preparations with some exogeneous proteases, among which chymotrypsin and pronase were especially effective. Further experiments with protease inhibitors have confirmed that alkaline band formation was due to TK limited degradation occurring during cell extraction. According to individual specificity of the inhibitors employed it has been proposed that a relative increase in the levels of Ca^{2+}-independent cysteine-protease activities of AD fibroblasts could be the most reasonable explanation for TK alterations. The latter appear to be strictly related and confined to this peculiar neurological disfunction and therefore useful as a biochemical marker of Alzheimer disease.

Materials and methods

Cell cultures and preparation of extracts

Primary cultures of forearm fibroblasts were obtained from the dermal biopsy of patients affected by Alzheimer's disease and from age-matched healthy control subjects. Cells were grown in DMEM (Gibco) supplemented with 10% FCS (Boehringer) at 37 °C in 5% CO_2 humidified atmosphere. Unless specified, each culture was usually splitted in three parts every 15 days, using a trypsin solution (0.5 mg/ml) for detachment. Confluence was reached within 7–10 days from inoculation and final cell density was around 5×10^4 cell/cm^2.

To prepare cell extracts, fibroblasts were washed three times with PBS in a bench centrifuge and pellets were lysed by ultrasonics (100 W, 20 sec) in the presence of 10 mM cold Tris-HCl buffer at pH 7.4 (about 5×10^6 cell/ml buffer). The lysates were centrifuged at 11000 rpm for 2 min (Beckmann Minifuge) in Eppendorf tubes and used for the analysis.

In experiments with commercial proteases, these were incubated in the extraction buffer at a concentration of 5 μg/ml for 15 min at 37 °C with the cell extracts. Proteases used were: chymotrypsin (40 U/mg, Sigma), trypsin (13000 U/mg, Sigma), papain (1000 MCU/mg, Calbiochem), pronase (10–20 U/mg, type XXVIII, Sigma) and thrombin (3250 U/mg, Calbiochem). Incubation of proteases with pure TK preparations from human leukocytes (Mocali and Paoletti 1989) were carried out using 0.5 μg protease and 25 μg TK (approximately 10 U/mg protein) in 1 ml of extraction buffer.

PMSF (phenylmethylsulphonyl fluoride), APMSF (4-amidophenyl methanesulphonyl fluoride), aprotinin, bestatin, chymostatin, EDTA, E-64 (*N-N*-L-3-*trans*-carboxyoxyrane-2-carbonyl-L-leucyl-agmantine), leupeptin, phosphoramidon were used as protease inhibitors and were added to the extraction buffer at the concentrations suggested by the purchaser (protease inhibitors set, from Boehringer Mannheim, Germany) as listed in Table 2.

Assays

Protein concentration was determined according to the method of Bradford (1976).

Isoelectric focusing (IEF) and immunostaininq of TK

The isoelectrophoretic separation of the enzyme was carried out in a Multiphor LKB apparatus equipped with a constant power supply. Gel slabs were prepared with 1% agarose IEF (Pharmacia, Bromma, Sweden) containing 5.4% ampholine solution (Pharmalyte 3–10, Pharmacia) and were run for about 1 h at 1 W/cm, using sulfuric acid (0.05 M) and sodium hydroxide (1 M) as the anode and cathode buffers, respectively. Separated proteins were transferred onto nitrocellulose by Southern capillary blotting and the enzyme was revealed by incubating the membrane with anti-TK rabbit IgG preparations followed by biotinilated anti-rabbit antibodies and by the avidin/biotin-immunoperoxidase system (Vectastain ABC kit, Vector Labs.) as reported previously (Mocali and Paoletti 1989).

The formation of the pH gradient on the gel was assessed by running a mixture of p*I* standards (pH range 3–10, Pharmacia).

Results and discussion

Human TK from cultured forearm fibroblasts can be separated by means of IEF into several forms migrating at distinct pH values (Paoletti et al. 1990). The normal enzyme pattern, as specifically detected with polyclonal anti-TK antibodies, consists of a major band around pH 8.4 and several minor forms migrating within pH 7.4–8.2 (Fig. 1, control). The reason for human TK microheterogeneity is not known; nevertheless it must be due to a well-established and physiological process since very similar profiles were obtained from the analyses of either intact or freshly-extracted fibroblasts (Paoletti et al. 1990) as well as of other mesenchymal cells, namely granulocytes and lymphocytes purified from peripheral blood (Mocali and Paoletti 1989).

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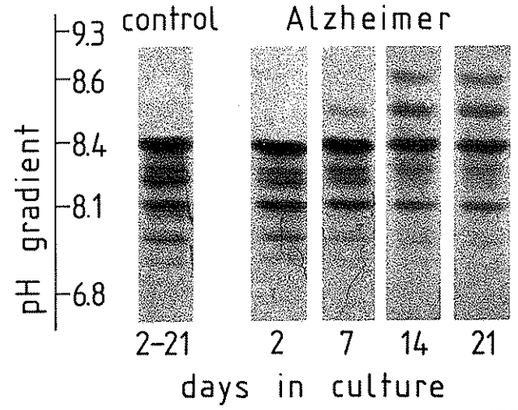

Fig. 1. Time-course of appearance of TK alkaline bands in the IEF profiles of extracts from cultured normal or AD fibroblasts. Cells were seeded at a density of about 10^4 cells per cm^2 and detached at 2, 7, 14 and 21 days of culture without medium changes. Washed cells were stored as a pellet at −80 °C until analyzed. Aliquots (40 μl) of the sonicated extracts (about 1 mg protein/ml of 10 mM Tris-HGl, pH 7.4) were applied to the agarose gel at 2 cm from the anode and submitted to IEF for 1 h at 10 °C. Southern blotting and immunostaining of TK bands was carried out as indicated in Materials and methods. The generation and the extent of pH gradient was assessed by running a p*I* standard mixture (pH range 3–10, Pharmacia).

Considering the constancy of the enzymic pattern the fact that the IEF profile of TK from fibroblasts of many AD patients presented peculiar and frequent alterations does seem intriguing. These alterations consisted of the appearance of enzyme forms having unusually high alkaline p*I* values and therefore called alkaline bands. However, despite the high frequency of TK alterations certain variations were observed. These concerned the spreading, intensity and number of TK alkaline bands among cell lines derived from different patients and also within individual samples taken from the same fibroblast cell line at different passages and times of culture.

This problem was investigated by evaluating the dependence of alkaline bands expression on culture conditions. Particularly we were interested to seek for changes in the TK pattern of control and AD fibroblasts along with incubation in culture without medium changes. The resulting IEF profiles (Fig. 1) indicated that there were striking changes in the expression of alkaline bands in cells analyzed at 2, 7, 14 and 21 days of culture. AD fibroblasts from a 2-day culture did not show any detectable TK abnormality, as compared to control cells. Evidence for some enzyme alterations began to appear in AD cells collected after 7 days of culture when a faint band became visible in the alkaline region of the immunoblot. Moreover, fibroblasts incubated for 14 and 21 days exhibited the full expression of TK alterations, consisting of two dark immunoreactive enzyme forms migrating within a pH range of 8.5–9. The appearance of alkaline bands was concomitantly accompanied by a decrease in staining of the two minor TK forms located between pH 7.5–8. This finding has been confirmed by three experiments carried out with three distinct cell lines from different AD patients. Besides, no alterations were observed in samples of control cultures treated according to the same schedule within the interval 2–21 days.

Further investigation were undertaken to assess whether the appearance of TK abnormalities correlated directly with the increase in cell density in the petri dish or with the time elapsed in culture. To this purpose a fixed amount of fibroblasts (10^6 cells) were inoculated in three dishes of different sizes (18, 25 and 78.5 cm^2), to obtain cultures which were hyperconfluent, confluent and subconfluent, respectively. Fibroblasts, collected two days later and analyzed for TK, exhibited three overlapping IEF profiles, lacking of any alkaline bands and identical to that obtained with control cells of Fig. 1 (data not shown). This fact seems to rule out the possibility that the expression of TK alterations might depend on cell crowding and contact inhibition or on the stage of cell growth. Rather it suggests that metabolic events leading to the generation of enzyme abnormalities in AD fibroblasts were closely linked to processes taking place along with "aging" of resting cultures.

Results reported elsewhere (Paoletti et al. 1990) have suggested that TK alkaline bands of AD fibroblasts occurred during cell extraction and might arise from enzyme limited degradation not operating similarly in normal cells. To verify this hypothesis fibroblast extracts from control cultures were treated with some commercial proteases (5 μg/ml), namely chymotrypsin, trypsin, papain, pronase and thrombin. The aim of the experiment was to check for protease-induced TK alterations, possibly resembling the alkaline bands detected in the IEF profile of AD fibroblasts. Indeed, some of the proteases (Table 1) were capable to cause partial TK degradation and converted the normal TK profile into the AD-like TK profile. The most striking effect was obtained with chymotrypsin which yielded two clearly stained alkaline bands, but also pronase and at a less extent papain were found to be positive. On the contrary, no alterations were produced by the action of trypsin and thrombin on normal cell extracts.

The generation of TK alkaline forms by proteases could be explained taking into account two different mechanisms: (a) commercial proteases activate endogenous cellular proteases which in turn will degrade TK and yield alkaline bands by an indirect process; (b) commercial proteases act directly on TK present in the extracts. The latter hypothesis was tested in a more controlled system where the whole cell extract was

TABLE 1

EFFECT OF SOME COMMERCIAL PROTEASES ON THE IEF PROFILE OF TK FROM NORMAL HUMAN FIBROBLASTS

Fibroblast extracts (about 1 mg protein/ml) from control cultures were incubated for 15 min at 37 °C with or without commercial proteases (5 μg/ml final concentration). Aliquots (40 μl) of each sample were submitted to IEF and TK was revealed as reported in Materials and methods.

	Appearance of alkaline bands
Untreated fibroblast extract	–
Extract plus chymotrypsin	+ + +
Extract plus trypsin	–
Extract plus papain	+
Extract plus pronase	+ +
Extract plus thrombin	–

TABLE 2

EFFECTS OF PROTEASE INHIBITORS ON THE APPEARANCE OF ALKALINE BANDS IN THE IEF PROFILE OF TK FROM ALZHEIMER FIBROBLASTS

Collected AD fibroblasts were suspended in 10 mM Tris buffer pH 7.4 with and without the listed protease inhibitors at the given concentrations. Following extraction, aliquots (40 μl) of each sample (about 1 mg protein/ml) were submitted to IEF and immunostaining for TK as reported in the Materials and methods.

	Appearance of alkaline bands
AD fibroblasts (untreated)	+ + +
AD fibroblasts plus PMSF (0.5 mM)	+ +
AD fibroblasts plus APMSF (25 μ mM)	+ + +
AD fibroblasts plus aprotinin (2 μg/ml)	+ + +
AD fibroblasts plus bestatin (40 μg/ml)	+ + +
AD fibroblasts plus chymostatin (10 μg/ml)	–
AD fibroblasts plus E-64 (1 μg/ml)	–
AD fibroblasts plus leupeptin (5 μg/ml)	±
AD fibroblasts plus phosphoramidon (0.2 mg/ml)	+ + +

replaced with pure TK preparations to be challenged with the above mentioned commercial proteases. Owing to the unavailability of pure enzyme from human fibroblasts we employed TK isolated from human leukocytes which presents the same IEF enzyme profile (Mocali and Paoletti 1989) and has common mesenchymal origin of fibroblasts. Results reported in Fig. 2 showed that the incubation of TK with papain (b), pronase (c) and especially chymotrypsin (d) (0.5 μm/ml, each) induced a partial hydrolysis of TK yielding alkaline bands. Besides, it is worth mentioning that due to the purification procedure the profile of isolated TK showed already a partial degradation as revealed by the darkening of the low p*I* bands while they

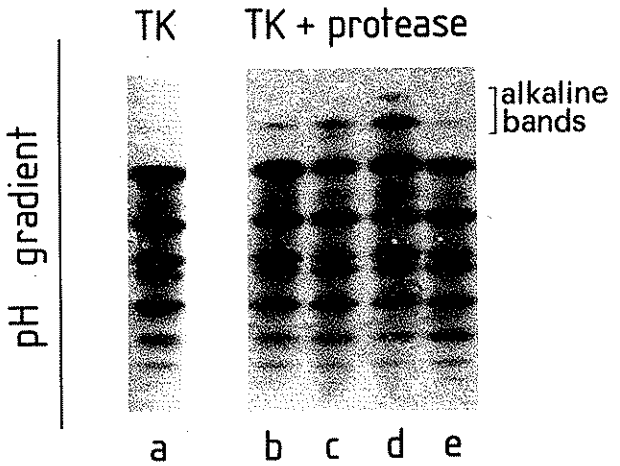

Fig. 2. Effect of commercial proteases on the IEF profile of pure TK preparations. Solutions of pure TK (25 μg protein/ml) from human leukocytes (Mocali and Paoletti 1989) were incubated for 15 min at 37 °C in the absence (a) or in the presence of commercial proteases, namely papain (b), pronase (c), chymotrypsin (d) and trypsin (e), at a final concentration of 0.5 μg/ml. Aliquots (20 μl) of each incubation mixture were then submitted to IEF and TK was detected by immunostaining as indicated above.

are faint in the fresh extracts. Nevertheless the IEF profile of untreated purified enzyme preparations never exhibited alkaline bands. These were generated from TK by the direct action of chymotrypsin and pronase to suggest that these enzymes mimic quite well effects of endogenous proteases which should be operating in AD fibroblasts while they kept silent in control cells.

A partial identification of the class of enzymes to which typical TK degradation might be ascribed, was eventually provided by experiments with a panel of protease inhibitors. These were added to AD fibroblasts prior to cell extraction to test their relative abilities in inhibiting TK proteolysis and preventing alkaline band formation (Table 2). The protective effect was maximum with E-64 (1 μm/ml) which is a specific inhibitor of cysteine-proteases and with chymostatin (10 μm/ml) which inhibits chymotrypsin and some cysteine-proteases (Umezawa 1976). A decrease in alkaline bands was also evident with leupeptin which is an inhibitor of both serine and cysteine-proteases. No protection was exerted by 25μM APMSF whereas some effect was obtained with PMSF but at a concentration (0.5 mM) much higher than those required for inhibition of serine-protease activities. Aprotinine (serine-protease inhibitor), bestatin (aminopeptidase inhibitor) and phosphoramidon (metalloprotease inhibitor) at the listed concentrations did not prevent alkaline band formation. According to the relative specificity of each inhibitor the intracellular cysteine-proteases appeared to be the class of endogenous proteases most competent for TK limited degradation.

Eventually, alkaline bands were equally produced in the presence of 0.5 mM EDTA, while the addition of 2

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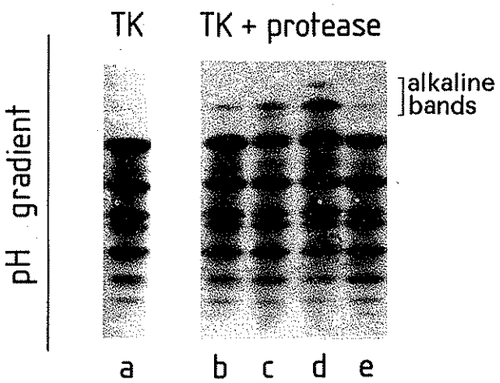

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TABLE 2

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Eventually, alkaline bands were equally produced in the presence of 0.5 mM EDTA, while the addition of 2 mM Ca^{2+} reduced at a certain extent TK abnormalities (data not shown).

Concluding remarks

TK alkaline bands seem to be the result of enzyme limited degradation carried out by a class of endogenous proteolytic enzymes we have tentatively identified as Ca^{2+}-independent cysteine-proteases exhibiting chymotrypsin (pronase)-like activity. Cultured AD fibroblasts, as compared to normal and other pathological cells, appeared to contain relatively enhanced levels of these proteases which were liberated and/or activated only at the time of cell extraction yielding the typical TK Alzheimer pattern. Whether TK limited degradation was actually due to a real increase in proteases or to a decrease in inhibitors remains to be established.

Moreover, TK alterations were virtually absent in freshly-plated AD fibroblasts while well detectable in "aged" resting cultures where intracellular degradative processes were likely to be increased. Eventually, alkaline bands were produced in the presence of EDTA indicating that neither metallo-proteases nor cytosolic calpains were involved in the process. All these evidences might be consistent with the conclusion that TK serves as the substrate for cysteine-proteases possibly located in the lysosomal compartment. TK may not be the only or even the preferred protein to be degraded. However, alkaline bands represent so far a reliable marker closely associated with the disease and might provide also clues to the biochemical defect in AD. It may also be worth mentioning that TK profiles of about 80 distinct leukocyte samples from chronic alcoholics have never shown abnormalities resembling AD alkaline bands (manuscript in preparation).

Current hypotheses on the pathogenesis of AD point to an imbalance of proteolysis which would promote neuronal loss and amyloid deposition in the brain of affected individuals (Ponte et al. 1988; Tanzi et al. 1988; Kitaguchi et al. 1988). Among the several evidences supporting this view there are reports concerning abnormal proteolytic cleavage of amyloid-precursor protein (APP) (Esch et al. 1990), altered protein processing (Selkoe 1989; Zhang et al. 1989), and the increase in lysosomal proteases (Cataldo and Nixon 1990) in autopsy brain. The latter finding, in particular, seems to match exceedingly well with our results leading to the suggestion that similar enhancement in proteolysis might occur in both nervous and non-nervous tissues. This would imply that cultured AD fibroblasts could be used as a model for investigating the biochemical defect whose comprehension is so far hampered in autopsy brain.

We have reported that cysteine-proteases responsible for TK limited degradation exhibited chymotrypsin (pronase)-like activity. Without further data we cannot interpret this fact; however we can at least emphasize that protease nexin II (soluble form of APP) (Khang et al. 1987), abundantly present in the plaque almyloid deposits (Glenner and Wong 1984; Masters et al. 1985), has been identified as a potent anti-chymotrypsin inhibitor (Abraham et al. 1988; Van Nostrand et al. 1989). This appears to be a very intriguing coincidence deserving deeper investigation.

Acknowledgments This work was supported by grants (60 and 40%) of the Ministero della Pubblica Istruzione. Authors are grateful to Dr. M. Marchi for his help in TK purification and analysis.

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