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Metabotropic glutamate receptors and brain ischemia: differential effects on phospoinositide turnover in young and aged rats

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Summary. - Neuronal damage and phosphoinositide hydrolysis stimulated by neurotransmitter receptor agonists in cerebral cortex of 3- and 24-month Fischer 344 rats, following an episode of brain ischemia, were compared. Transient global ischemia was induced by occlusion of common carotid arteries for 15 minutes in conditions of moderate hypoxia. Seven days after, histological examination of cerebral cortex showed cell loss, neurons with nuclear pyknosis, cytoplasmatic degeneration, and glial proliferation. Ischemic lesions appeared moderate to severe in young rats and intermediate in all the aged animals. In young rats inositol phosphates accumulation stimulated by excitatory amino acids (ACPD, ibotenate and quisqualate), but not by norepinephrine or carbachol, was enhanced significantly with respect to sham-operated animals. No potentiation at all was observed in aged rats. This finding suggests that the events leading to the increased metabotropic response in the post-ischemia period is impaired by the ageing process.

Key words: global brain ischemia, inositol phosphates accumulation, cerebral cortex, ageing, rat.

Riassunto *(Recettori metabotropi del glutammato e ischemia cerebrale: effetti sul "turnover" dei fosfoinositidi in ratti giovani e vecchi).* - Nel presente lavoro sono stati analizzati il danno neuronale e l'idrolisi dei fosfoinositidi, dopo stimolazione con agonisti dei recettori metabotropi, nella corteccia cerebrale di ratti Fischer 344 di 3 e 24 mesi, a sette giorni dall'induzione di un'ischemia globale transitoria. L'esame istologico evidenziava picnosi, degenerazione citoplasmatica, scomparsa di elementi cellulari neuronali e proliferazione gliale, di entità da moderata a severa nei ratti di 3 mesi ed intermedia in tutti i ratti di 24 mesi. Un significativo aumento dell'accumulo di inositolo fosfato indotto dagli amino acidi eccitatori, ma non dalla norepinefrina e dal carbacolo, era evidente solo negli animali giovani rispetto agli sham-operati. Tali dati suggeriscono l'esistenza di una alterazione età-dipendente dei parametri che regolano la risposta metabotropa nel periodo post-ischemico durante l'invecchiamento.

Parole chiave: ischemia cerebrale globale, accumulo di inositolo fosfati, corteccia cerebrale, invecchiamento, ratto.

Introduction

In the last decade evidence has accumulated showing that excitatory amino acids (EAA) receptors are involved in brain ischemic and postischemic events: the overstimulation of ionotropic glutamate (iGlu) receptors contributes to the ischemic brain damage [1-3], while metabotropic glutamate (mGlu) receptors linked to phosphoinositide (PI) turnover (i.e., group I mGlu receptors) appear to be mainly involved in post-ischemic phenomena. Indeed, the agonist-stimulated PI hydrolysis in hippocampal and cortical slices from young adult Wistar rats was shown to be higher in post-ischemic animals than in controls [4].

Recently we have reported a similar post-ischemic enhanced response of mGlu receptors to EAA in cortical slices from young adult Sprague Dawley rats [5]. Since stroke in humans is primarily a disease of middle or advanced age, in the last years the relevance of using aged, rather than young, animals in experimental research on cerebral ischemia has been stressed [6, 7]. Therefore, we considered interesting to assess whether, following ischemia, there are age-related differences in EAA-elicited PI hydrolysis in the cerebral cortex. On this purpose we compare such a response, 7 days after the ischemic insult, in young and aged Fischer 344 rats; this strain being preferred since it appears to be commonly used in ageing research and more suitable than Sprague Dawley [8, 9]. Moreover, the brain lesions were evaluated histologically and immunohistochemically.

Experimental procedures

Induction of ischemia

Male rats of the Fischer 344 strain (Charles-River, Calco, CO, Italy) of two age groups (i.e., young = 3 to 4 months, and aged = 23 to 25 months, weighing 250 to 270 and 350 to 380 g, respectively) were anesthetized with *equithesin* (chloral hydrate 4.2%, sodium pentobarbital 1%) injected i.p. (4 ml/kg). Eighteen young and twenty old rats were submitted to transient global cerebral ischemia as previously described [5] by occluding for 15 minutes both common carotid arteries (CCA) and keeping the animal in moderately hypoxic conditions (15% O_2). Body temperature was maintained at 37 °C with a lamp placed above the animal and the blood pressure controlled. Seven control rats of each age were sham-operated, i.e. anesthetized, their neck incised, and CCA exposed. All animals were then returned to their home cage and maintained in standard conditions (22 ± 1 °C and 50 ± 10% relative humidity) until sacrifice. Thirteen rats of each groups survived 7 days after ischemia. The directives of the Council of the European Communities (86/609/EEC) on animal care were duly observed.

Histology and immunohistochemistry

Six ischemic rats from each experimental group were used for histology and immunohistochemistry. Seven days after the ischemic insult they were anesthetized and brains fixed by perfusion with 4% formaldehyde buffered to pH 7.4, removed, dehydrated in graded alcohol, cleared with xylene and embedded in paraffin. Subsequently, sagittal brain sections 8 µm thick were cut with a microtome and stained with hematoxylin-eosin and cresyl violet.

Immunostaining for glial fibrillar acidic protein (GFAP) was performed on deparaffinized, rehydrated sections by the indirect streptavidinbiotin peroxidase assay, using a commercially available kit (DAKO-LSAB, Dako SpA, Milan). Sagittal 8 µm sections were incubated in a moist chamber at 37 °C for 45 min in GFAP antiserum raised in rabbit. The antiserum was diluted 1:100 in 0.1 M sodium phosphate buffer, pH 7.2. The sections were then incubated at room temperature for 10 min in biotinylated anti-rabbit antibodies. Streptavidin peroxidase solution was then added using 3% of 3-amino-9-ethylcarbazole in N,N- dimethyl-formamide as chromogen. Counterstaining was performed with Mayer's hematoxylin [5].

Evaluation of PI hydrolysis

Seven ischemic and seven sham-operated additional rats from each age group were used for measuring the PI cellular response. They were killed by decapitation 7 days after the ischemic episode or the sham operation, their brains removed, the cortex dissected out and then sliced in 350 x 350 µm sections with a McIlwain chopper. The slices were prelabelled with myoinositol (New England Nuclear Corporation, Boston, USA) for 60 min, rinsed, and the mGlu receptor agonists added at concentrations producing the maximal H-inositol phosphates (IP) accumulation, (0.5 mM 1S,3R-trans ACPD or 0.5 mM ibotenate or 0.005 mM quisqualate); in order to assess whether the enhancement of PI response was specific for EAA, muscarinic and adrenergic agonists (i.e., 1 mM carbachol and 0.1 mM norepinephrine) were also used. After 60 min incubation in the presence of lithium, PI hydrolysis was measured as IP accumulated, as previously described [10, 11]. All assays were carried out in triplicate. Basal IP accumulation was expressed in DPM/tube, while agonist-elicited response as the DPM stimulated/basal ratio. Significance of differences between the two age groups was assessed by Student's t-test.

Results

Histology and immunohistochemistry

Histological and immunohistochemical analysis showed evident ischemic injury in cerebral cortex, particularly in the parietal area. The cortical damage was moderate in three young rats (limited to layers V and VI, with scarse loss of neurons, some with swollen cytoplasm and central chromatolysis, and a weak glial reactivity, Fig. 1a) and severe in the others (extending also to layers II, III and IV, with massive cell loss and surviving neurons showing swollen and scalloped cytoplasm; a marked glial proliferation surrounded the damaged area, Fig. 1b). Injury in all the aged rats, particularly evident in layers II, V and VI, was of intermediate severity (with moderate cell loss, damaged neurons showing shrunken cytoplasm and pyknotic nucleus; marked glial proliferation was also present, Fig. 1c). GFAP immunostaining of damaged parietal cortex (Fig. 1d-f) showed pronounced proliferation of astrocytes with considerably enlarged cell bodies and processes, particularly evident in severely damaged young rats and in aged ones (Fig. 1e, f).

PI hydrolysis

As shown in Fig. 2, seven days after the ischemic insult the EAA-elicited PI response in the brain cortex of Fischer-344 young rats was significantly increased as compared to that of sham-operated animals of the same age. On the other hand such a response in aged ischemic rats was unchanged with respect to controls. The enhancement of EAA-stimulated PI response in

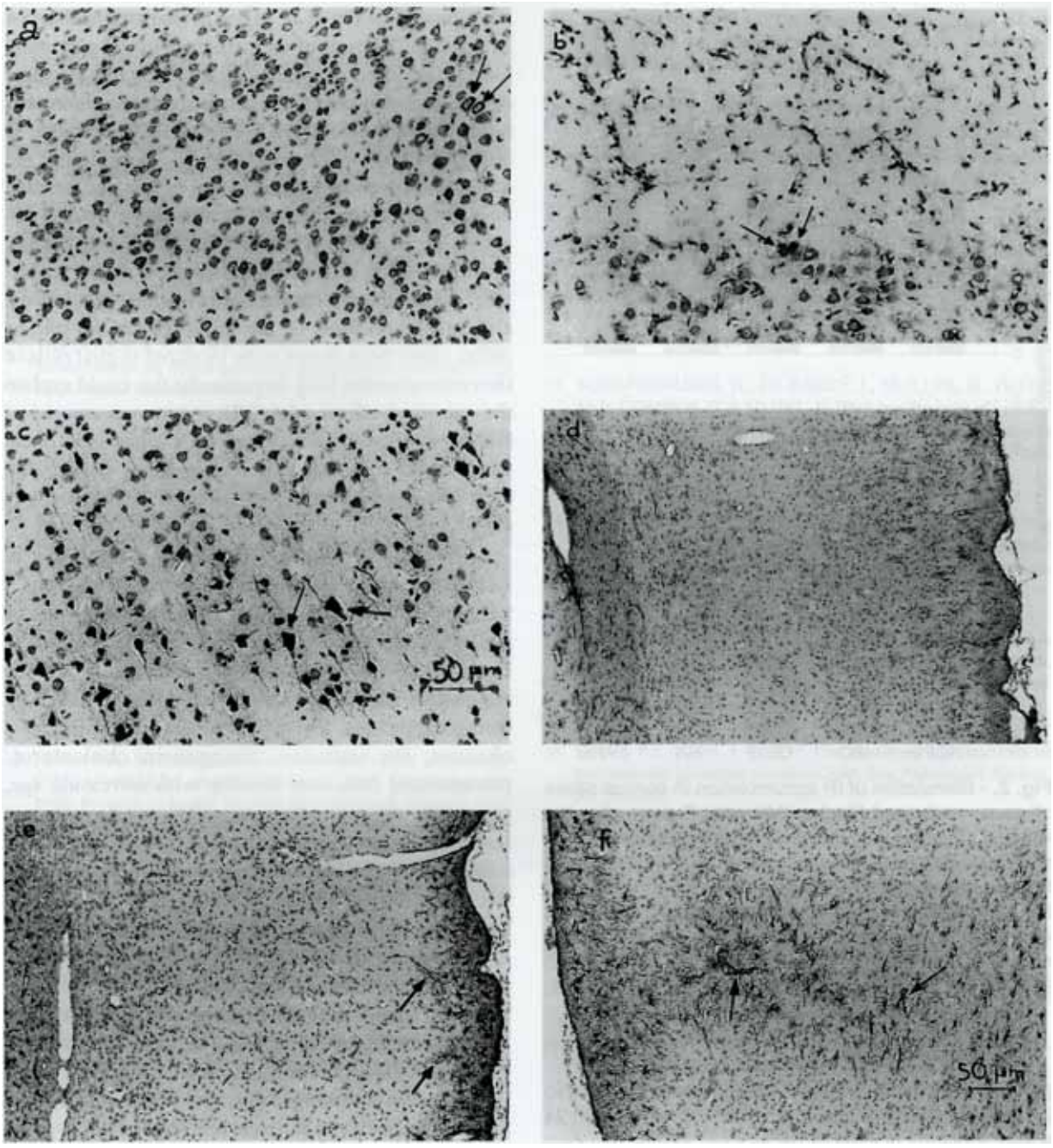

Fig. 1. - Sagittal sections, 8 µm thick , of parietal cerebral cortex of ischemic young adult and aged Fischer 344 rats showing typical neurodegeneration, neuronal necrosis and glial proliferation, 7 days after the ischemic insult. **(a)-(c)** staining with cresyl violet. Magnification 300 x: **(a)** parietal cerebral cortex of a young rat showing moderate neuronal damage. In some neurons central chromatolysis is visible (arrows); **(b)** parietal cerebral cortex of a young rat showing severe neuronal damage. Surviving neurons exhibit scalloped cytoplasm (arrows); **(c)** parietal cerebral cortex of an aged rat showing neuronal damage of intermediate severity. Damaged neurons with shrunk en cytoplasm are visible (arrows). Some nuclei appear pyk notic (arrow head). **(d)-(f)** immunostaining for GFAP (counterstained with hematoxylin). Magnification 200 x: **(d)** Layer V of parietal cerebral cortex of a moderately damaged young rat showing proliferation and moderate hypertrophy of astrocytes; **(e)** layer V of parietal cerebral cortex of a severely inj ured young rat showing, in the surrounding damaged area, intense proliferation and hypertrophy of astrocytes (arrows); **(f)** layer V of parietal cortex from an aged rat showing intense proliferation and hypertrophy of astrocytes (arrows).

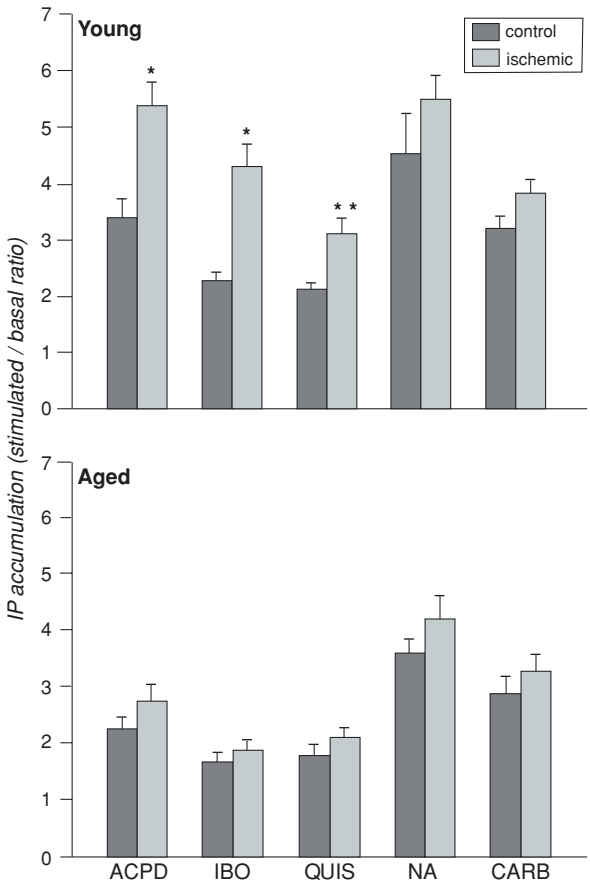

Fig. 2. - Stimulation of IP accumulation in cortical slices of young and aged Fischer 344 rats, 7 days after the ischemic insult. Concentrations of the agonists (mM): ACPD 0.5, IBO 0.5, QUIS 0.005, NA 0.1, CARB 1.0. Basal IP accumulation (in DPM/tube): 404 ± 63 and 398 ± 73 for control and ischemic young rats, 420 ± 78 and 464±122 the respective values for aged rats. Values are means ± SEM from 7 independent experiments carried out in triplicate, for both young and aged rats. * * $p < 0.001$, * $p < 0.01$ control*vs* ischemic (Student's t-test).

post-ischemic rats appears then to be age-dependent. No increase in agonist-induced PI hydrolysis was found 24 h post-ischemia in none of the two age groups (data not shown). In addition, a decreasing trend of the control response was present in aged respect to young rats. The PI response to carbachol and to norepinephrine both in young and in aged rats was unchanged 7 days after the ischemic insult. This suggests that in Fischer 344 rats the effects of ischemia on PI hydrolysis are rather specific for mGlu receptors agonists.

Discussion

The potentiation of EAA-elicited PI response found post-ischemia in young Fischer 344 rats is consistent with our previous data on Sprague Dawley rats [5] and with those of Seren *et al*. [4] on Wistar strain. The last authors, on the basis of their findings on ^{3}H-glutamate binding, excluded an up-regulation of the active receptors density following ischemia, and postulated an increased efficiency of the coupling between mGlu receptors, G protein and phospholipase C (PLC). Considering the intense post-ischemic proliferation of glia, the positive modulation of group I mGlu receptors by the co-activation of group II mGlu receptors [12-14] might contribute to the increased PI response if such a "cross-talk" is hypothesized to occur also in glial cells. Indeed, also mGlu receptors of the group II ($mGlu_2$ and/or $mGlu_3$) have been shown to be localized in glial cells of the cerebral cortex [15]. In particular this could explain the enhanced effects of ACPD, a common agonist of both group I and II mGLu receptors; it appears less likely with respect to quisqualate and ibotenate considering their low agonist potency on group II mGlu receptors [16].

A finding of interest of the present study is that in ischemic aged rats the PI response remained unchanged with respect to controls. This indicates that in spite of evident neuronal damage and marked glial response, the cascade of events leading to the increased metabotropic response in the post-ischemic period was impaired by the ageing process. The nature of such an impairment is obscure; for instance, changes in cholesterol/phospholipid ratio may develop with increasing age, concomitantly, membrane viscosity may be impaired resulting in an altered receptor/signal transduction relationship [17, 18]. Both the possible enhancement of the coupling of mGlu receptors to PLC and the hypothesized "cross talk" between glial mGlu receptors of group I and II would result then compromised. The decreased responsiveness of cortical mGlu receptors to agonists found in aged Fischer rats in a previous investigation of our laboratory [19], and confirmed in the present experiments by the decreasing trend of the PI response in control aged rats, could contribute to make them less sensitive to the ischemic insult.

A rather unexpected and somewhat intriguing result of the present investigation is the fact that the PI response in young rats, independently of the severity of ischemic damage, was always enhanced, while in aged rats, in spite of evident cortical lesions, such an enhancement was absent. This would suggest that neuronal damage is not reflected in the post-ischemic PI response. Further experiments are in progress using a technique of magnetic resonance imaging (MRI), recently standardized in our laboratory [20], which makes possible to localize in the same animal damaged brain areas and in these areas to evaluate PI hydrolyis. This will facilitate the assessment of any likely relationship between the two phenomena.

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Characterization and screening of a point mutation in LDL receptor gene found in Southern Italy ($FH_{Avellino}$)

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Summary. - The finding that the missense mutation C331W in the exon 7 of LDL-receptor gene, previously reported to occur in Holland and Belgium, caused the homozygote familial hypercholesterolemia (FH) in an individual from the district of Avellino induced us to search the mutation in a large area of region Campania. This was made with simple screening methods developed by ourselves and based on either the recognition of a primer-induced Fok I restriction site in the mutant allele or the PCR allele-specific amplification (PASA) of mutant allele. They were applied to a total of 144 unrelated cases recruited from where the mutation was more likely to occur. We failed to reveal any new case of C331W mutation that is indeed not common within the area of this screening, at spite of having been found in different countries.

Key words: mutagenic primers, mutation detection, familial hypercholesterolemia, low density lipoprotein receptor, PCR allele-specific amplification.

Riassunto *(Caratterizzazione e ricerca di una mutazione puntiforme nel gene del recettore LDL individuata nell'Italia meridionale, $FH_{Avellino}$)*. - La mutazione C331W nell'esone 7 del gene del recettore LDL già descritta in soggetti belgi e olandesi è stata da noi individuata, in forma omozigote, in una famiglia originaria dell'avellinese. Ciò ci ha indotto a ricercare la mutazione in soggetti affetti da ipercolesterolemia familiare provenienti da una vasta area che include e circonda la zona del ritrovamento iniziale. A tale fine sono state messe a punto due semplici procedure analitiche di amplificazione PCR, una che con un primer mutagenico introduce un sito di restrizione Fok I nell'allele mutante, l'altra che con un opportuno *primer* amplifica specificamente l'allele mutante. Le metodiche sono state validate e applicate a un totale di 144 soggetti senza legami di parentela. Il fatto che non siano stati trovati nuovi casi suggerisce che la mutazione sia rara, almeno nell'area studiata, nonostante sia stata individuata in nazioni diverse.

Parole chiave: ipercolesterolemia familiare, mutazioni genetiche, recettore LDL, analisi di mutazioni, amplificazione PCR.

Introduction

Familial hypercholesterolemia (FH) may be caused by a plethora of different mutations at the LDL-receptor (LDLR) locus [1]. Therefore, the development of simple detection methods is crucial to the genetic diagnosis of the disease. This task is generally easy in the case of mutations that alter the restriction pattern of the affected gene, but may be quite difficult in the other cases. The good results previously obtained with the detection of LDLR gene mutations in Italy by either PCR allele-specific amplification (PASA) [2] or methods based on the primer-induced restriction enzyme recognition site (i.e. mismatch or mutagenic PCR amplification) [3] encouraged us to improve these simple techniques and to extend their use to Italian mutations that need simple detection procedures.

This report describes the characterization of a point mutation, identified by sequence analysis, in a family from the Avellino district in southern Italy. Precisely, it was the 1056C>G transversion, causing the missense Cys>Trp at codon 331 (TGC>TGG, C331W) in the exon 7 of the LDLR gene that was named FH-Avellino-1, from the origin of the family. It was previously reported in two separate families in Holland and Belgium respectively [4, 5] but never found in Italian FH individuals. The presence elsewhere of this mutation and the finding of a homozygous individual in an Italian family suggested to extend its search with simple screening methods based, as above described, on the primer-induced restriction site and the PCR allele specific amplification. The tests developed appeared reliable and were used for examining a large sample of FH individuals. Surprisingly they did not reveal any new

unrelated case. However, the PASA technique appeared to be the simpler and cheaper approach for conducting further screenings of the C331W mutation.

Materials and methods

Patients and controls

The study started from the affected family. Then, it was extended, using the two screening methods described, to 32 healthy controls, 50 unrelated FH index cases recruited from where the mutation was more likely to occur (i.e. the area of region Campania including and surrounding the district of Avellino), and 94 unrelated FH index cases recruited from the nearest areas of southern Italy. All patients and controls gave their informed consent to blood sampling and to genetic study.

Analytical methods

DNA extraction was performed from peripheral leukocytes of FH patients and control donors following standard procedure [6]. The mutation was detected by single-strand conformational polymorphism analysis (SSCP) of the exon 7 following its PCR amplification according to Hobbs *et al*. [1]. The SSCP analysis was performed by a non-radioactive procedure previously described [7]. Briefly, 250 ng of genomic DNA were amplified in a total volume of 50 µl with 10 mmol/l Tris-HCl, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 100 µmoles of each of the four nucleotides, 10 pmoles of each primer and 2 units of Taq DNA polymerase (Promega). An aliquot of the PCR product was diluted three-fold in loading buffer (98% deionized formamide, 10 mM NaOH, 0.02% bromophenol blue and xylene cyanol). The mix was heat denatured (94 °C for 2 min), snap chilled on ice and fractionated at room temperature by using a vertical gel unit with 10% polyacrilamide gel (99:1, acrylamide:bis). After electrophoresis the bands were visualized with the following silver stain protocol: the gel was sequentially stirred in 10% ethanol for 3 min, 1% nitric acid for 3 min, 0.1% silver nitrate for 15 min, rinsing twice with distilled water between each step. Finally, the colour was developed with the mix 6% sodium carbonate - 0.1% formaldehyde until the desired intensity was achieved (3-5 min). The development was stopped with 10% acetic acid. The sample showing an abnormal SSCP migration pattern of exon 7 was sequenced by using an automated fluorescent ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) following the manufacturer's recommendations. The mutation identified by the automated sequencer was confirmed by manual sequencing as previously described [8], using the appropriate primers for exon 7 according to Hobbs *et al*. [1].

We developed two different approaches for the rapid detection of the mutation. One was based on the primer-induced restriction enzyme recognition site (or mismatch PCR amplification) in the gene section involved by the mutation. The other was based on the allele-specific amplification of the mutant allele (PASA). The mismatch amplification was carried out with the primer pair: 5'-AGTCTGCATCCCTGGCCCTGCGCAG-3' (forward primer) and the 5'- GCTCAGTCCACCGGGG-ATTCACCAT-3' (reverse primer). These allowed the amplification of a 167 bp section of LDLR gene encompassing the exon 7 (NCBI accession number: L00341) and spanning from the last 25 bases of intron 6 to the first 22 bases of intron 7. The intronic sequences for the design of these primers were downloaded from a public domain database (http://www.ucl.ac.uk/fh/). The forward primer exactly matches the sequence of wild allele [1]. The reverse primer introduces an A by T change in the base preceding the 3' terminus of the primer. This creates a Fok I restriction site (GGATG 9/13) in the amplification product of the mutant allele, but not of wild allele. The working principle of this method is illustrated in Fig.1 (panel A). In practice the PCR mix included: 250 ng of genomic DNA, dNTPs (200 µM each), Taq DNA polymerase Promega (1 U) and its buffer containing $MgCl_2$ (2.0 mM in the final mix), primers (20 pmoles each), in a final volume of 50 µl. PCR reaction was carried out for 30 cycles in a Perkin Elmer termal cycler mod. 2400. After an initial denaturation at 94 °C for 5 min, each cycle consisted of denaturation

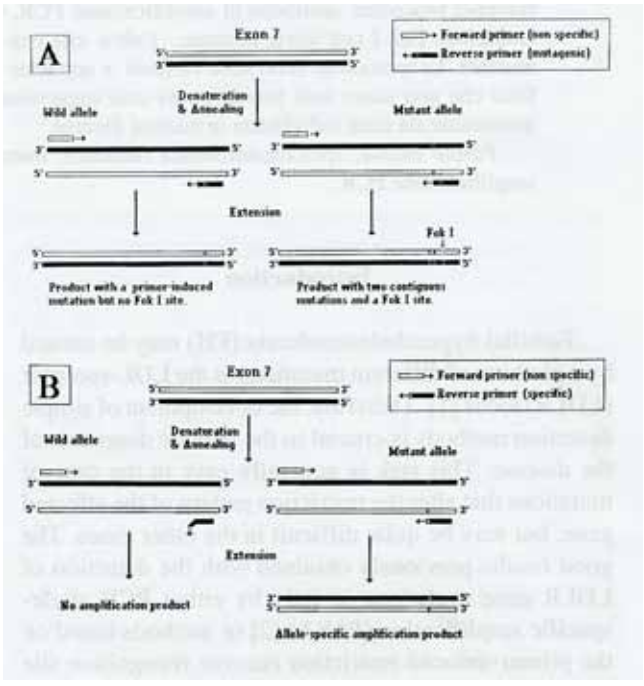

Fig. 1. - Work ing principles of the methods tested. Panel A: method based on the primer-induced restriction enzyme recognition site. Panel B: method based on PCR allele-specific amplification.

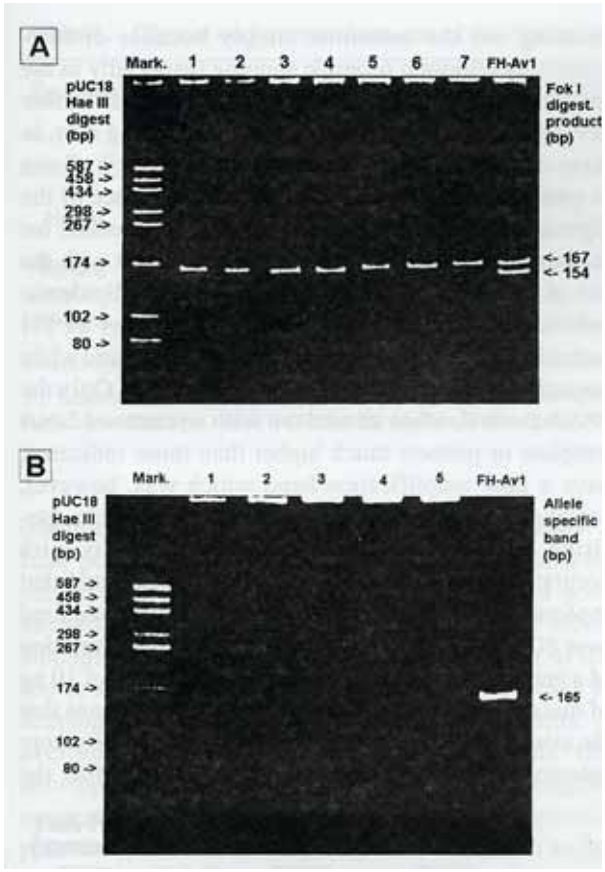

Fig. 2. - Examples of application of the methods tested. Panel A: method based on the primer-induced restriction enzyme recognition site. Lanes 1 to 7: DNA samples from FH probands. Lane mark : pUC18 Hae III digest used as a molecular weight mark er. Lane FH-Av-1: DNA from a heterozygous individual with the C331Wmutation. Panel B: method based on PCR allele-specific amplification (lanes as above).

for 30 s (94 °C), hybridation for 30 s (65 °C) and elongation for 1 min (72 °C). PCR ended with a final 72 °C extension for 7 min. After PCR, 20 µl of the amplification product was mixed with 4 U of the restriction enzyme Fok I (Boehringer Mannheim), supplemented with 2.5 µl of the specific incubation buffer and water up to 25 µl final volume. After overnight incubation at 37 °C the digestion product was electrophoresed on 12% polyacrylamide gel (or 2% agarose gel). The bands were stained with ethidium bromide and visualized with UV transilluminator. The amplification product (167 bp) in the case of the mutant allele was split into shorter fragments (154 + 13 bp), and in the case of wild allele remained unaffected (Fig. 2; panel A).

The allele-specific amplification (PASA) was performed with the same forward primer used in the above described method, but with a different reverse primer: 5'-GTCCACCACCGGGGATTCACCTTCC-3'. In this case, the reverse primer had a non-tolerated pairing (C/C) at its 3' end with the wild allele, but had the normal C/G pairing with the mutant allele. This prevented the amplification of wild allele, but not of mutant allele. The working principle of this method is illustrated in Fig. 1 (panel B). In practice the PCR mix included: 250 ng of genomic DNA, dNTPs (200 µM each), Taq DNA polymerase Promega (1 U) and its buffer containing $MgCl_2$ (1.5 mM in the final mix), primers (10 pmoles each), in a final volume of 50 µl. PCR reaction was carried out for 30 cycles on the following conditions: after an initial denaturation at 94 °C for 7 min, each cycle consisted of denaturation for 30 s (94 °C), hybridation for 30 s (58 °C) and elongation for 1 min (72 °C). PCR ended with a final 72 °C extension for 7 min. After PCR, 10 µl of the amplification product was electrophoresed on 10% polyacrylamide gel. The bands were stained with ethidium bromide and visualized with UV transilluminator. The mutant allele gave an amplification product of 165 bp, the wild allele did not show any amplification product (Fig. 2; panel B).

The haplotype of some relevant intragenic polymorphisms, i.e., Stu I in exon 8, Hinc II in exon 12, Bst EII in intron 12, Ava II in exon 13, Apa LI 5' in intron 15, Pvu II in intron 15, Hae III in exon 18, Nco I in exon 18, and the alleles of microsatellite D19S394, 0.15 cM upstream from the gene, were determined with methods previously described [9-14]. However, the routine evaluation of the Ava II polymorphism was performed by a simple SSCP method developed by ourselves. Briefly, a 217 bp fragment encompassing exon 13 was amplified under standard PCR conditions with the primer pair described by Hobbs *et al.* [1]. An aliquot of the amplification product was subjected to heat denaturation and silver staining as above described for SSCP analysis. The single-strand product was electrophoresed at 5-8 °C on a mini gel vertical apparatus (Hoefer Scientific, 8 x 10 cm slabs) with 10% polyacrylamide gel (99:1, acrylamide:bis). The different genotypes gave clearly identifiable patterns, as shown in Fig. 3.

Results and discussion

This mutation was initially detected in a homozygous individual from apparently non related parents by a SSCP screening of the different exons of LDLR gene. Exon 7 showed an abnormal SSCP pattern (Fig. 4) that sequence analysis indicated to be due to a C by G transversion at nt 1056.

Table 1 reports the clinical findings of the proband and his parents, and the haplotype co-segregating with the mutation. The mutation C331W, located in the Repeat A of the EGF-precursor homology domain, most likely alters the receptor folding and leads to a receptor-defective phenotype.

The screening with the method based on the primer-induced restriction enzyme recognition site was conducted with 80 unrelated index cases. The screening

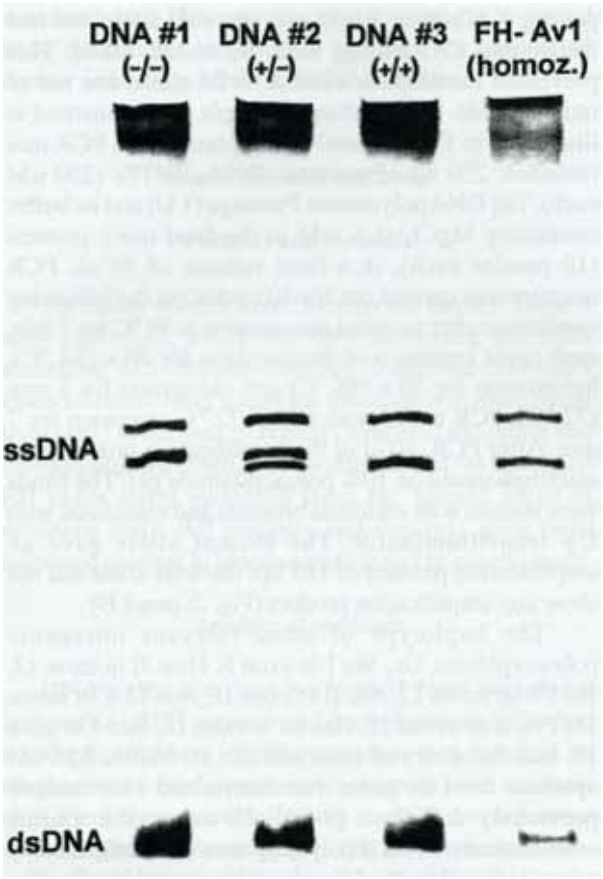

Fig. 3. - Genotyping of Ava II polymorphism by SSCP analysis. Lanes DNA no. 1, 2 and 3: samples characterized for the Ava II polymorphism by restriction analysis and used as a reference for SSCP analysis. Lane FH-Av1: DNA sample from a C331W homozygous demonstrating the co-segregation of this mutation with the existence of AVA II restriction site (i.e. + haplotype).

with the method based on allele-specific amplification was conducted on 64 additional unrelated index cases. In 20 out of the 144 cases both screening methods were applied. However, no new subject with the mutation was identified. Therefore, the C331W did not appear to be a common mutation, neither in the affected family's area nor in the other areas considered. Studies reporting the same mutation abroad do not mention the ethnic origin of the affected subjects, preventing us from speculating on a possible common origin of the defect. Both the methods used appeared suitable for conducting wide-scale screening of the mutation. However, some specially devised measures must be adopted to guarantee the reliability of these methods. The method based on primer-induced restriction enzyme recognition site in many cases yielded a weak amplification band, depending on the quality of DNA templates. In these cases the examination of the restriction enzyme digestion product may lead to a difficult identification of the band pointing out the mutation, simply because of low-sensitivity ethidium bromide staining (especially in the case of heterozygous samples). We found that further development of the gel with the silver- staining may, in these cases, circumvent this difficulty because it allows to establish without doubt the eventual presence of the digestion product. Both the methods were checked for the possibility of giving a false positive result with the use of 32 control DNA samples from normolipidemic individuals originating from the same areas as FH individuals. We never observed any equivocal band while operating under the conditions here described. Only the PASA method, when carried out with amounts of DNA template or primers much higher than those indicated, gave a faint amplification band which was, however, easily distinguished from that formed by a genuine positive sample. Specifically, a series of tests with accurately controlled DNA samples demonstrated that the formation of a false positive (but weak) band required over 800 ng of wild DNA template, while the formation of a genuine band was already evident at levels of 10 ng of mutant heterozygote DNA template. This means that the allele-specific primer is about a hundred times more selective for mutant than for wild allele and that the

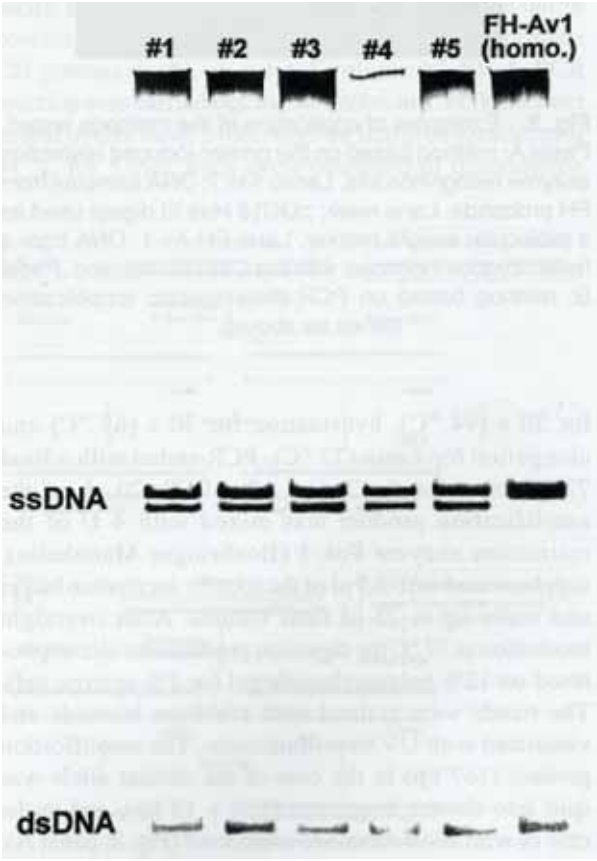

Fig. 4. - Alteration of SSCP pattern in the amplification product of exon 7 of LDLR gene by the C331Wmutation. Lanes no.1 to no.5: DNA samples from normolipidemic individuals. Lane FH-Av1: DNA sample from a C331W homozygous individual.

Table 1. - Clinical and genetic features of the family carrying the C311Wmutation

Zygosity	Sex	Age (years)	LDL chol. (mmol/l)	Xanthomas	Cardiovascular involvement	LDL-R activity (% of controls)	Treatment
Homo	M	9	16.65	Tu, Te	Normal angiogram	9	LDL-apheresis
Hetero	M	39	4.91	-	IHD	-	Statins
Hetero	F	30	5.89	-	-	-	Statins

Tu: tuberous xanthomas; Te: tendon xanthomas; IHD: ischemic heart disease.
Haplotype: Stu I (+), Hinc II (-), Bst EII (-), Ava II (+), Apa LI 5' (-), Pvu II (+), Hae III (+), Nco I (+).

conditions described for the PASA test do not allow a false positive result. This was further demonstrated by the lack of positive cases in tests conducted with normolipidemic subjects, even with doubled DNA amounts. On the other hand, this high sensitivity of the test to minute amounts of mutant DNA template is in keeping with the absence of false negative results. A secondary but interesting point concerns the development of the allele-specific primer. According the suggestions given in other reports an allele-specific primer requires a non-tolerated mismatch (such as the A/G or C/C or A/A mismatches) between its 3' end and the base subject to mutation in the wild template [15]. We verified this by trying the four different primers possible when the base at 3' end was changed in the oligonucleotide sequence of the allele-specific primer. The results of these tests performed with samples from both wild and mutant homozygous genotype are summarized in Table 2. They indicate that only the C/C mismatch allows the amplification of mutant allele but not of wild allele. The A/C mismatch, even if not included in the non-tolerated mismatches, appeared most effective in preventing the amplification. This is probably to be taken into account in the development of allele-specific primers for other mutations. This allows also to exclude, at least in theory, that other mutations known to affect the same codon, i.e., the C331Y (or 1055G>A) found in Mexico [1] and the C331X (or 1056C>A) found in Poland [16], may lead to false C331W positives by both the proposed methods.

Table 2. - Result of the PCR allele-specific amplification under the conditions described of either wild or homozygote C331Wmutant DNA with allele-specific primers differing only in their 3' end

Primer 3' end	Wild allele pairing/amplification		Mutant allele pairing/amplification	
C	C/C	no	C/G	yes
G	G/C	yes	G/G	yes
A	A/C	no	A/G	no
T	T/C	yes	T/G	yes

In conclusion, the PASA method appeared the method of choice in view of further screenings of the C331W mutation. This in consideration of its reliability, rapidity, and costs, being not necessary the digestion with a restriction enzyme of PCR amplification product.

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