Cytotoxic activity of a recombinant GnRH-PAP fusion toxin on human tumor cell lines

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Received 15 February 2000; received in revised form 28 March 2000

Edited by Veli-Pekka Lehto

Abstract Pokeweed antiviral protein (PAP), a ribosome-inactivating protein isolated from the leaves of *Phytolacca americana*, reveals potent antiviral activity against viruses or cytotoxic action against cells once inside the cytoplasm. Therefore PAP is a good candidate to be used as an immunotoxin. We constructed a bacterial expression plasmid encoding PAP as a fusion protein with gonadotropin-releasing hormone (GnRH), a neuropeptide with receptor sites on several gynaecologic tumors. The resulting recombinant toxin was produced in *Escherichia coli* and accumulated in inclusion bodies. After purification under denaturing conditions, renaturated GnRH-PAP shows an IC_{50} of 3 nM on in vitro translation assays and selectively inhibits the growth of the GnRH receptor positive Ishikawa cell line (ID_{50} of 15 nM); on the other hand, neither GnRH nor PAP alone had any effect.

Key words: Pokeweed antiviral protein; Recombinant chimeric toxin; Gonadotropin-releasing hormone; Immunotoxin

1. Introduction

Immunotoxins and recombinant toxins are cytotoxic agents designed to selectively kill populations of cells that display specific cell surface antigens. These toxins are developed mainly for cancer therapy [1]. They are also employed for the treatment of other diseases such as acquired immunodeficiency syndrome (AIDS) [2] and are useful as tools in neurobiology [3]. They are composed of a targeting moiety (antibody, growth factor or hormone) linked to a bacterial or a plant toxin (Pseudomonas exotoxin, diphteria toxin or ribosome-inactivating proteins e.g. ricin or pokeweed antiviral protein) by chemical coupling or recombinant DNA technology [4–7].

Pokeweed antiviral protein (PAP) is a ribosome-inactivating protein produced by the plant *Phytolacca americana* [8]. This enzyme is an RNA *N*-glycosidase which specifically removes an adenine residue from a highly conserved loop in the large ribosomal RNA of eukaryotic and prokaryotic ribosomes [9,10], inducing a conformational change in the subunit. This prevents the binding of the elongation factor EF2, thus blocking protein synthesis. PAP has been classified as an antiviral agent because it reduces the infectivity of many plant viruses when co-inoculated with a virus on the leaves of susceptible species [11,12]. Ready et al. have shown that PAP is sequestered in the cell wall matrix and have proposed a model for the mechanism of its antiviral activity [13]. Their hypothesis has been corroborated with the demonstration of PAP having activity against pokeweed ribosomes [14]. Thus, PAP could be released in the cytoplasm of infected cells, together with the virus, and exert its toxic activity on the ribosomes. Virus replication would be thereby prevented by a local suicide. The transport pathway of this powerful toxin (IC_{50} of 1 nM on pokeweed ribosomes) synthesized at a very high yield (up to 0.5% of soluble leaf proteins) [14] has not been yet elucidated. Our group has recently identified an inactive complex form of PAP called PAPi [15]. Although the composition of PAPi is still under investigation, hypothetically PAPi may be a self-protection mechanism of pokeweed cells against PAP which might be misaddressed during its biosynthesis. The amount of PAP versus complexed PAP (in PAPi) can be estimated in a plant extract with an ELISA which discriminates between the two forms [16]. PAP has also been shown to be highly toxic in vitro to cells infected with different animal viruses including the human immunodeficiency virus (HIV) [17–19]. Unlike type II ribosome-inactivating proteins (RIPs, e.g. ricin) or bacterial toxins (which are able to penetrate living cells through their cell recognition domain), PAP is not cytotoxic. This protein, which contains four cysteine residues involved in two intramolecular disulfide bonds, is not glycosylated. Consequently, PAP represents an excellent candidate for the toxic moiety of an immunotoxin. It has already been chemically conjugated to different antibodies. It has been used in a preclinical study for treatment of B lineage leukemia/lymphoma [20], against HIV-infected, CD4-positive cells [21] and as a purging agent in the case of human autologous bone marrow transplantation [22]. More recently, Dore et al. have produced in *Escherichia coli* a recombinant hybrid toxin which combines the interleukin 2 (IL2) with a mutant form of PAP. This mutant form, enzymatically active against eukaryotic ribosomes, allowed *E. coli* growth [23].

The gonadotropin-releasing hormone (GnRH), also called luteinizing hormone-releasing hormone (LH-RH), is a neuro-

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Abbreviations: GnRH, gonadotropin-releasing hormone; PAP, pokeweed antiviral protein; RIPs, ribosome-inactivating proteins; IC_{50}, concentration of inhibitory protein yielding 50% of inhibition in the rabbit reticulocyte lysate translation system; ID_{50}, lethal dose of inhibitory protein yielding 50% of viability of a population cell in a proliferation assay; PAGE, polyacrylamide gel electrophoresis; LB, Luria Bertani

PII: S0014-5793(00)01469-1

decapeptide. After binding to its receptor on pituitary cells, it triggers the secretion of the luteinizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropin hormones control the activity of the gonads. A growing interest has developed for the application of GnRH and related peptides in the fields of gynaecology and oncology. GnRH and its analogues have been used in treatment of hypogonadism and infertility. It is also used in medical castration for the treatment of gonadotropin dependent cancers [24].

Moreover, GnRH receptors have been identified on tumor cells from mammary, endometrial, prostatic and pancreatic tissues as well as on tumor cell lines [25–28].

For these reasons, GnRH is a very good candidate for the engineering of recombinant chimeric toxins. Furthermore, this peptide binds to its receptor with high affinity (K_d about 10^{-9} M) [29] and is not immunogenic.

In this paper, we report the elaboration of a chimeric toxin associating PAP and the GnRH by recombinant gene technology; this recombinant protein is toxic towards the endometrial cancer cell line Ishikawa harboring the GnRH receptor.

2. Materials and methods

2.1. GnRH-PAP construction and expression

The cDNA encoding the recombinant PAP was cloned in the expression vector pOPE90 as previously described [30]. The GnRH-PAP fusion cDNA was amplified by PCR. The sense DNA primer (5′-CAg gat cc**C AGC ACT GGT CCT ATG GAC TGC GCC CTG GA***G CCA AGA AAC TGA ACG ACG CTC AGG CGC CGA AGA GTG AT*A TGG TGA ATA CAA TCA TCA TCT AC-3′) introduced a *Bam*HI restriction site to facilitate the cloning (lower case), the full sequence of GnRH (bold), followed by the staphylococcal protein A FB fragment sequence (italic) and a sequence encoding the eight first amino acids of mature PAP (upper cases) [31,32]. The FB fragment sequence corresponded to the residues 48–60 of the fragment B of the staphylococcal protein A (FB) and served as a linker to improve the efficiency and the solubility of the fusion protein [31]. The full length cDNA of PAP except for the 22 codons, corresponding to N-terminal signal peptide, served as a template for PCR amplification.

The antisense primer (5′-CGC gga tcc GAA TCC TTC AAA TAG AT-3′) was designed to introduce a *Bam*HI restriction site (lower case) at the 3′-end of the PAP gene. The PCR-amplified sequence was extracted from agarose gel using the 'Cleanmix' kit (Talent, Italy). After digestion with *Bam*HI, it was ligated to *Bam*HI site of the pQE60 expression vector (Qiagen) to make the pQE60/GnRH-PAP construction (Fig. 1). These ligation products were used to transform rubidium chloride competent JM 109 bacterial cells. The recombinant plasmid was purified from LB culture medium containing ampicillin (50 μg/ml) and sequenced. Then, it was employed to transform rubidium chloride competent M15 bacterial cells containing the pREP4 plasmid. Bacterial growth was performed with overnight culture in LB medium containing ampicillin (50 μg/ml) and kanamycin (25 μg/ml). Once the LB culture medium absorbance reached 0.5 at 600 nm, the GnRH-PAP fusion protein expression was induced by the addition of 1 mM IPTG for 2 h at 37°C.

2.2. Purification of the GnRH-PAP recombinant toxin

After centrifugation (2000×*g*, 5 min, 4°C), the bacterial pellet was suspended in buffer A (50 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.2 mM β-mercaptoethanol, 0.05% Tween 20) 5 ml/g of wet weight, sonicated (10 bursts at 4°C) and centrifuged (8000×*g*, 10 min, 4°C). The supernatant was discarded. The inclusion bodies were suspended in buffer B (8 M urea, 0.1 M sodium phosphate, 0.2 mM β-mercaptoethanol, 0.01 M Tris–HCl, pH 8) sonicated for 1 min and incubated in the buffer for 1 h at room temperature under gentle shaking. The solution was cleared by another centrifugation (10 000×*g*, 10 min, room temperature) and was applied on a Ni-NTA column (1 cm×10 cm, Qiagen) equilibrated with buffer B at a flow rate of 0.1 ml per hour. Proteins bound to the column by non-specific interactions were removed with buffer C (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris, pH 6.3, 0.2 mM β-mercaptoethanol, imidazole 10 mM) and the chimeric protein was eluted from the column with buffer C in the presence of 250 mM imidazole. The purified protein was detected in the elution fractions by SDS–PAGE. For the refolding, the GnRH-PAP containing fractions were pooled, diluted 5 times and dialyzed 3 times against 5 l of 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 0.2 mM β-mercaptoethanol at room temperature for 48 h. The total protein content was estimated by the method of Lowry [33].

2.3. Western blotting analyses and immunoprecipitation

The proteins were treated under denaturing conditions (sample buffer: 62.5 mM Tris–HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 5% β-mercaptoethanol (v/v), 0.02% bromophenol blue (w/v)) and separated by SDS–PAGE according to Laemmli [34]. The gels were stained with Coomassie R250 blue dye. For Western blotting analysis, the proteins were transferred from the gel onto PVDF membrane using a Bio-Rad trans-blot apparatus as described by Towbin [35]. After 1 h blocking in PBS–BSA buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1% of BSA) at room temperature, the membrane was incubated for 2 h with the culture supernatant containing anti-PAP monoclonal antibodies [36]. After washing with PBS buffer, containing 0.05% Tween 20 and 0.5 M NaCl, the membrane was incubated for 30 min with alkaline phosphatase conjugated goat anti-mouse antibodies (Biosys, France) which was diluted 1/2000 in PBS–BSA buffer. After extensive washing, immunoreactive bands were visualized with a substrate solution containing nitroblue tetrazolium chloride (NBT, 0.33 mg/ml) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP, 0.165 mg/ml, Promega) in 0.1 M Tris–HCl buffer, pH 9.5 containing 0.1 M NaCl and 5 mM $MgCl_2$.

Purified recombinant GnRH-PAP (5 μg) was immunoprecipitated either with 3 μg of polyclonal anti-GnRH (a generous gift from Prof. D. Fellmann, UPRESA CNRS 6025 Besançon, France) or with polyclonal anti-PAP antibodies in Tris buffer 100 mM pH 7.4 (final volume 100 μl). Forty microliters of protein A Sepharose (Sigma) equilibrated in the same buffer were added and the mixture incubated for 1 h at room temperature under gentle agitation. The protein A Sepharose was recovered by centrifugation (12 000×*g* 15 min, room temperature) and washed twice with PBS containing 0.5 M NaCl. The immunoprecipitated proteins were removed from protein A with SDS–PAGE sample buffer and analyzed by Western blotting using monoclonal anti-PAP antibodies for the detection.

2.4. Inhibition of in vitro translation

Different concentrations of GnRH-PAP or PAP alone (2 μl) were added to the translation mixture. The latter contained 4 μl of rabbit reticulocyte lysate (Promega), 0.2 μl of a mixture of different amino acids (minus methionine) at a concentration of 1 mM, 0.2 μl of L-[^{35}S]methionine (3×10^8 Bq/ml, Dupont) and 5.2 μl of H_2O. The reaction was started by the addition of 0.2 μl of a stock solution of brome mosaic virus (BMV) RNA at 0.5 mg/ml. After incubation (1 h at 30°C), 2 μl aliquots of each assay were analyzed by SDS–PAGE. Finally, the gel was autoradiographed. Densitometric analyses of the band of 35 kDa encoded by BMV RNA was performed with the Bio-Rad 'Gel-Doc' System.

2.5. Detection of GnRH receptor expression on cell lines

Expression of the GnRH receptor was checked on various cell lines by RT-PCR and Western blotting.

To perform RT-PCR analyses, total RNAs from rat pituitaries (a generous gift from Professor D. Fellmann, UPRESA CNRS 6025 Besançon, France) and from cultured cell lines were extracted by the guanidium isothiocyanate method according to Chomczinski and Sacchi [37]. Complementary DNA was synthesized with a Moloney murine leukemia virus reverse transcriptase (Life Technologies). The reaction mixture (25 μl) contained 5 μg of total RNA, 10 μM of oligo-dT primer, 1×RT buffer, a mixture of each dNTP 0.5 mM and 0.5 units of enzyme; it was incubated for 1 h at 37°C. PCR and nested PCR were performed on the cDNA with two sets of primers as described by Imaï et al. [28].

For Western blotting analyses, cellular membrane enriched fractions prepared according to Wiznizer et al. [38] were separated by SDS–PAGE, transferred onto PVDF membranes and revealed with an anti-GnRH receptor antibody (Santa-Cruz).

2.6. Cytotoxicity

Ishikawa cells were a generous gift from Dr L. Bermont (IETG). The human breast cancer cell line MCF-7 was kindly provided by Dr S. Saez (Centre hospitalier Lyon Sud France). The cells were maintained in Dulbecco's modified medium (Sigma) supplemented with penicillin, 100 U/ml, streptomycin, 100 μg/ml, amphotericin B, 0.25 μg/ml penicillin (Sigma) and 10% fetal calf serum. All cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37°C and the medium was replaced every 2–3 days.

For cytotoxicity assays, the cells were seeded in 96-microwell plates (4000 cells/well) in the same medium to which various concentrations of GnRH-PAP were added. After 36 h of incubation, viability assay was performed by using the cell proliferation kit II (XTT) (Roche Diagnostics).

3. Results

3.1. Expression and purification of recombinant GnRH-PAP

After induction of M15 *E. coli* cells, the recombinant GnRH-PAP, mainly contained in inclusion bodies, was purified to homogeneity by Ni^{2+}-NTA-chelate affinity chromatography at a yield of 1 mg/l of bacterial culture and identified as a 36 kDa protein by SDS–PAGE (Fig. 2A). Western blotting analyses allowed us to detect PAP moiety in the purified fraction at the same molecular weight as that of the stained purified protein (Fig. 2B). Immunoprecipitation analyses allowed the detection of the GnRH moiety in the GnRH-PAP fusion protein (Fig. 2C), thus confirming the presence of both the entities in the fusion product.

3.2. Analyses of in vitro toxicity of GnRH-PAP

As PAP inhibits in vitro protein synthesis, we tested if GnRH-PAP has a similar inhibitory activity. Fig. 3 shows that GnRH-PAP was able to inhibit protein synthesis in a dose dependent manner with an IC_{50} of 3 nM. A control assay indicated that purified PAP shows an IC_{50} of 0.5 nM (Fig. 3).

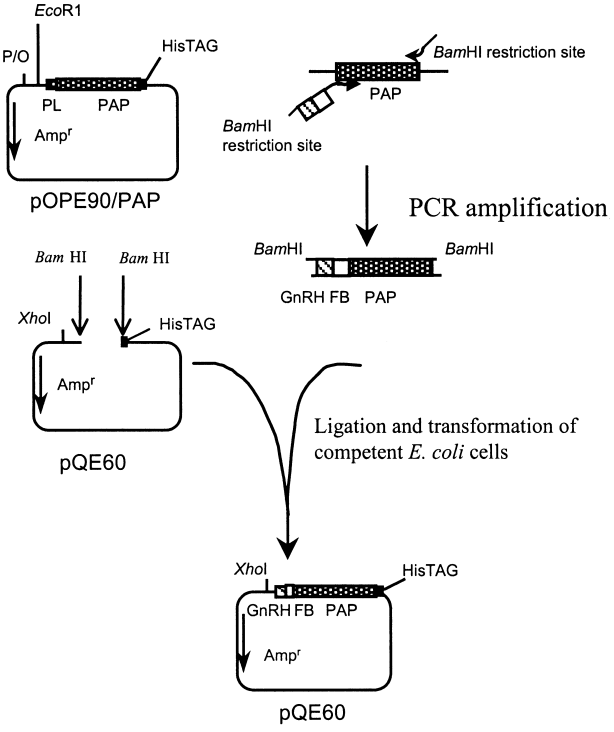

Fig. 1. Cloning strategy of the GnRH-PAP construct into the pQE60 expression vector. PL, pectate lyase leader sequence; P/O, promoter/operator sequence.

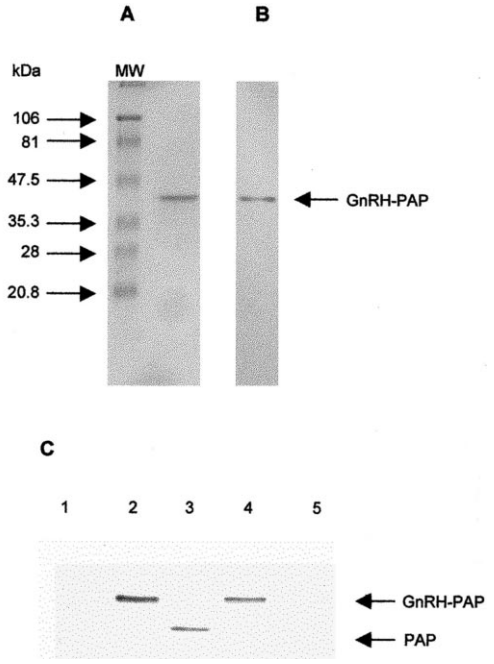

Fig. 2. Analysis of the GnRH-PAP purified protein. A: Detection of the GnRH-PAP fusion toxin after Ni^{2+}-NTA-chelate affinity chromatography. Ten microliters of purified fraction were loaded on a 12% denaturating polyacrylamide gel. The proteins were then stained with Coomassie brilliant blue. B: Western blotting analyses performed on the same purified fraction. The recombinant protein was revealed with anti-PAP and PAL conjugated anti-mouse monoclonal antibodies (Biosys, dilution 1/4000). C: Immunoprecipitation of the GnRH-PAP. GnRH was incubated with antibodies, immunocomplexes were then adsorbed on protein A Sepharose, run on SDS–PAGE and transferred as previously described. Immunological detection was performed with anti-PAP monoclonal antibodies. Lane 1, GnRH-PAP immunoprecipitated (IP) with no antibodies; lane 2, GnRH-PAP IP by anti-PAP antibodies; lane 3, PAP IP by anti-PAP antibodies; lane 4, GnRH-PAP IP by anti-GnRH antibodies; lane 5, PAP IP with no antibodies.

3.3. Detection of GnRH receptor in Ishikawa and MCF-7 cell lines

Prior to cytotoxicity analyses, GnRH receptor expression was checked both by RT-PCR and Western blotting analyses. From Ishikawa cell RNAs, RT-PCR experiments allowed us to amplify a 319 bp fragment similar to the one obtained with the control RNAs from pituitary extracts (Fig. 4). No such band was obtained from our MCF-7 cell line, although the histone H3.3 347 bp fragment was correctly amplified (Fig. 4) [39].

Western blotting analyses performed with a monoclonal antibody against the GnRH receptor confirmed the RT-PCR results: the GnRH receptor could only be detected in Ishikawa cell extract (data not shown). Moreover, immunofluorescence labelling experiments using FITC labelled GnRH indicated a relatively heterogeneous labelling on Ishikawa cells and no labelling on MCF-7 cells (data not shown).

3.4. Cytotoxicity of the GnRH-PAP fusion protein against cultured cells

The purified GnRH-PAP chimeric toxin was incubated with

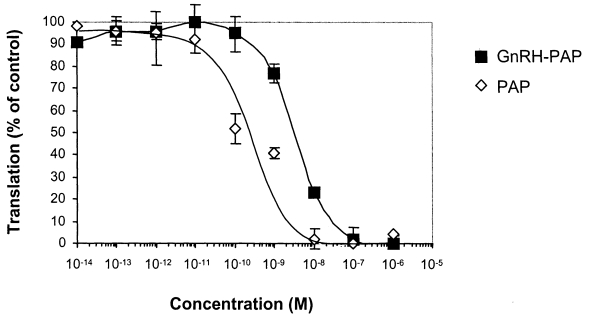

Fig. 3. Inhibition of in vitro translation. Rabbit reticulocyte lysates 5 μl were incubated with BMV RNA as template, [^{35}S]methionine, a mixture of amino acids and various concentrations of either GnRH-PAP or PAP. Then, 2 μl aliquots were run in a 12% SDS–PAGE and autoradiographed. In vitro translation rate was estimated from gel scans done with a phosphorimager (Bio-Rad).

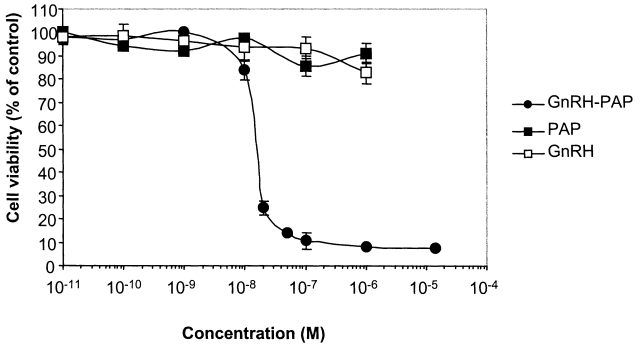

Fig. 5. GnRH-PAP toxic activity against cultured cells. Ishikawa cells were seeded in 96-microwell plates and maintained in complete medium at 37°C for 2 days. Then, various concentrations of either GnRH-PAP, GnRH or PAP were added. After 36 h of incubation, cell viability was estimated with the cell proliferation kit II (XTT) (Roche Diagnostics). Dot represents an average value (% of viability of the control) of eight replicates.

Ishikawa and MCF-7 cells in complete medium at concentrations ranging from 5×10^{-8} M to 10^{-12} M. Fig. 5 shows that GnRH-PAP was able to induce cell death of the Ishikawa cell line, whereas GnRH alone or PAP alone did not decrease the cell viability. The ID_{50} of GnRH-PAP was estimated to be 15 nM. MCF-7 cell cultures were not affected by equivalent concentrations of GnRH-PAP (Fig. 6). Control of GnRH-PAP specificity against Ishikawa cells was performed by incubating together GnRH-PAP (10^{-6} M) with free GnRH (10^{-5} M). The cell viability with GnRH-PAP was less than 10% of untreated control. Upon adding GnRH, it climbed up to 87%. This result argues in favor of the specificity of the GnRH-PAP fusion toxin.

4. Discussion

We have previously cloned and produced in high yields the PAP cDNA in *E. coli*. This allowed us to produce recombinant chimeric toxins combining PAP and cell binding ligands. In this paper, we report the cloning and the expression of a GnRH-PAP fusion construct. The cloning strategy was facilitated by the small size of the GnRH peptide (10 amino acids), thus permitting us to directly insert the GnRH sequence at the 5′-extremity of the PAP cDNA. The choice of the 5′-extremity of the PAP cDNA was dictated by the known importance of the C-terminal extremity for PAP cytotoxicity [16,40]. The resulting recombinant toxin GnRH-PAP was expressed and easily purified to homogeneity by a one step NI^{2+} affinity chromatography.

The presence of both PAP and GnRH moieties in the purified protein was asserted by two different techniques. PAP was easily detected by Western blotting analyses, whereas GnRH necessitated immunoprecipitation. In fact, the polyclonal anti-GnRH antibody provided by Professor Fellmann was not able to detect the GnRH with Western blotting analyses (D. Fellmann, personal communication). Thus, the presence of the GnRH moiety in the fusion protein was confirmed by immunoprecipitation experiments.

The chimeric toxin was able to inhibit in vitro protein synthesis with a relatively good efficiency but to a lesser extent than the purified native PAP or the recombinant PAP [30,41]. This slightly reduced activity could be due to a conformational change possibly due to the presence of the GnRH moiety in the N-terminus of the fusion product.

Prior to cytotoxicity experiments, the expression of the GnRH receptor was tested by RT-PCR and Western blotting analyses. We used a nested PCR in order to allow the detection of very low amounts of GnRH receptor messenger RNA. Moreover, these primers [28] allowed the amplification of a short sequence of the GnRH receptor cDNA. The same frag-

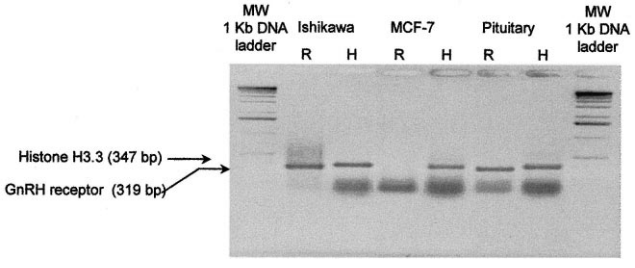

Fig. 4. Detection of gonadotropin-releasing hormone (GnRH) receptor mRNA in different cell lines. Following reverse transcription, the first-strand cDNA was amplified by polymerase chain reaction (PCR) using a set of oligonucleotide primers specific for the GnRH receptor mRNA. A second PCR was performed on the amplified DNA with internal primers yielding a 319 bp fragment (R, GnRH receptor). As a control, amplification of the histone H3.3 cDNA yielded a 347 bp fragment (H, histone). The cDNA was resolved on a 2% agarose gel.

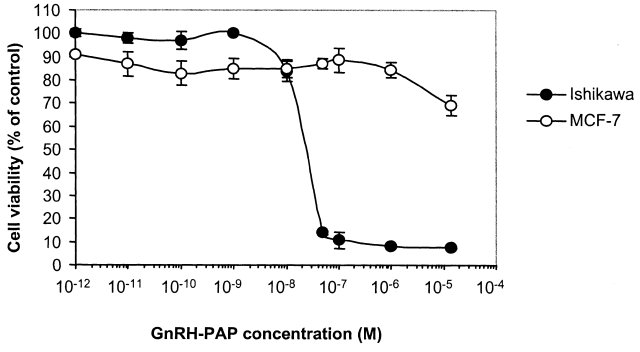

Fig. 6. Effects of GnRH-PAP on Ishikawa and MCF-7 cell lines. MCF-7 cells were seeded as described for Ishikawa cells and then incubated with equivalent concentration of GnRH-PAP. Viability tests were performed as previously described. Values represent an average of eight replicates.

ment could not be amplified from genomic DNA because of the presence of two introns (respectively 4353 bp and 4972 bp between the primers used for the first PCR cycle) [42]. Thus unspecific amplified products which would be provoked by DNA contamination were prevented. The GnRH receptor transcripts and protein were detected in the Ishikawa cell line as well as in the pituitary control; surprisingly they were absent in the MCF-7 cell line. The expression of the GnRH receptor has been previously reported in the two cell lines [25,26], however Chatzaki et al. failed to detect GnRH receptor in the Ishikawa cell line [43]. The lack of expression of the GnRH receptor in the MCF-7 cells might be a characteristic of our cell clone.

Cytotoxicity of GnRH-PAP was tested on the two tumor cell lines. The GnRH-PAP showed a very significant cytotoxicity on the Ishikawa cell line with an ID_{50} of 15 nM, whereas the fusion toxin had no effect on MCF-7 cells.

The GnRH moiety differs from the original hormone by the absence of the pyroglutamic acid residue no. 1. Moreover it is linked to PAP via a linker (FB). These differences could reduce the binding efficiency of the recombinant GnRH to its receptor, thus affecting the cytotoxicity of the fusion toxin.

When compared to an LH-RH-RNase A conjugate constructed by using the chemical cross-linking method (ID_{50}: 0.5×10^{-6} M) [44], GnRH-PAP ID_{50} appears very promising. However, it is lower than the values generally obtained with recombinant immunotoxins using *Pseudomonas* exotoxin (ID_{50}: 10^{-12} M) [45] or diphteria toxin (ID_{50} from 10^{-9} M to 10^{-12} M) [46]. The cytotoxicity is highly dependent on the targeting moiety and the number of ligand receptors on the cell surface. The need of a two step PCR amplification procedure indicates, in our case, that the GnRH receptors are poorly expressed in the Ishikawa cells; this may affect the cytotoxicity potency. The internalization and the rate of delivery of the toxin into the cytoplasm are also important factors. Nevertheless, it is likely that the binding capacity of GnRH-PAP could be improved by directed mutagenesis which permits the expression of GnRH analogs with greater affinity than in the case of the original GnRH [24,47]. The absence of GnRH-PAP cytotoxicity on MCF-7 cells corroborates the fact that, in these cells, the GnRH receptor was not detected either by RT-PCR or Western blotting experiments. Moreover, competition between free GnRH and GnRH-PAP on Ishikawa cells raised the cell viability from 10 to 87%. Hence, we have a good argument for the specificity of the GnRH-PAP to GnRH receptor positive cell lines.

In recent years, the discovery of specific antigens on tumor cells has been exploited to develop a new class of therapeutic agents which associate antibody with a toxin of bacterial or plant origin (formally known as immunotoxins). At present, the DNA recombinant gene technology allows the design of chimeric toxins which associate growth factors or other cell binding ligands with toxic moieties. Promising results have been obtained in clinical trials made with the new therapeutic agents: B cell or T cell malignancies can be treated with immunotoxins or chimeric toxins [48]. However, the treatment of solid tumors with these new therapeutic agents has been disappointing because of poor penetration of tumor mass, resulting in a loss of efficiency. Toxic side effects caused by non-specific cytotoxicity have previously been described [6,49,50]. These results compel researchers to explore new ways for obtaining new molecules of improved efficiency, smaller size and diminished non-specific cytotoxicity.

Very recent experiments have shown that tumor cells bearing GnRH receptors also expressed the 'death receptor' Fas [51]. The fixation of GnRH analogues on the GnRH receptors in these tumor cells induced apoptosis through the expression of the Fas ligand [52]. In the future, it will be interesting to investigate if the GnRH-PAP has the ability to induce apoptosis in targeted cells and to trigger the expression of Fas/Fas ligand system. The resulting amplified apoptosis would improve the efficiency of the GnRH-PAP fusion toxin on solid tumors.

Acknowledgements: We thank P. Millet and his colleagues at the Botanical Garden of the University of France-Comté, for providing the plant material; Professor D. Fellmann for the generous gift of the anti-GnRH polyclonal antibodies and the pituitary extracts, Dr L. Bermont for providing the Ishikawa cell line, Dr S. Saez for providing the MCF-7 cell line. We thank Dr. K. Lundstrom-Baudais for correcting the English. This work was supported by the Ministère de l'Education Nationale, de la Recherche et de la Technologie.

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