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ORIGINAL PAPER

The role of inflammation in the genesis of the cystic component of craniopharyngiomas

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Abstract
Background Craniopharyngioma accounts for 5–10% of childhood tumors and, despite of the benign histological features, its clinical course can be malignant because of critical anatomical relationships with neural and vascular structures and the possible morbidity associated to resection. Only a few studies have addressed the molecular characterization of the cyst fluid so far and the mechanisms of action of intracystic agents are not clearly understood yet.
Methods The acidic soluble proteins contained in the cystic fluid of six patients with cystic craniopharyngioma, three of them treated with intratumoral interferon-α, were analyzed. A high performance liquid chromatography electrospray ionization mass spectrometry analysis was performed.
Findings The antimicrobial peptides α-defensins 1–3 relevant for innate immunity were detected in the cystic fluid before the intratumoral treatment. Amount of peptides significantly decreased in cystic fluid during pharmacological treatment.
Interpretation Detection of α-defensins 1–3 excludes that cyst fluid formation can derive from disruption of blood–brain barrier and suggests the involvement of innate immune response in pathology of craniopharyngioma cyst formation. The reduction of α-defensins could derive both from direct antitumoral effect of interferon-α on squamous epithelial cells of craniopharyngioma cyst and from its immuno-modulatory effects on the recruitment of cells of innate immune systems. Interestingly, the clinical patient outcome well correlates with the gradual reduction of α-defensins 1–3 amount. Additional studies will be necessary to establish the role of these molecules in the pathogenesis of craniopharyngioma, and further investigations will be necessary to confirm the efficacy of the antitumoral activity of interferon-α.

Keywords Craniopharyngioma · Interferon-alpha · Innate immunity · Alpha-defensins

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Introduction

Craniopharyngioma accounts for 5–10% of childhood tumors and, despite of the benign histological features, its clinical course can be malignant because of the critical anatomical relationships with the neighboring neural and vascular structures and the possible morbidity associated to the surgical resection [9, 13].

Craniopharyngioma arises from ectopic remnants of the pharyngeal epithelium after the embryological evagination of the Rathke's pouch during development of the adenohy-

pophysis, taking on a metaplasic squamous epithelium [9, 18]. Due to the secretive properties of this epithelium, most part of craniopharyngioma has a cystic portion containing secreted fluid, cholesterol crystals, and epithelial cells [1]. The cystic component of tumor may be utilized to deliver chemo and radiotherapic agents, in order to control the solid tumor and reduce the volume of its cystic component with the main goal in the pediatric population to delay the surgical treatment in the pediatric population, consequently, allowing the patient to have a build development as normal as possible [3, 4].

The mechanisms of action of chemo and radiotherapic intracystic agents are not clearly understood yet. Interestingly, only a few studies have addressed the molecular characterization of the cyst fluid so far [2, 10, 12, 22, 25, 27, 28]. We have started an investigation aimed at characterizing the proteins/peptides content of the cystic craniopharyngioma in order to acquire information about molecular pathogenesis of the cyst formation and about the modifications its fluid content during and after the administration of intracystic interferon α (INF-α). The analysis was carried out by means of high-performance liquid chromatography coupled to ion-trap mass spectrometry with electrospray ionization (HPLC ESI-MS). In this preliminary report, we describe the level of α-defensive 1–3, a relevant component of cyst fluid, in six children with cystic craniopharyngiomas and the changes following the intracystic administration of INF-α in three of them

Methods

Patient and specimens collection

The cystic fluid samples were obtained from six patients affected by cystic craniopharyngioma (Table 1). They were four males and two females aged 3–6 years (mean 4.5 years). All patients performed a pre-operative magnetic resonance imaging (MRI) with gadolinium. The intracystic catheter was placed by a single burr hole through an endoscope-assisted technique in five cases and through a craniotomic approach in one case. Three patients underwent the intracystic treatment with IFN-α, that was administered via a subcutaneous Ommaya reservoir according to the protocol proposed by Cavalheiro et al. [4], that is 3.000.000 I.U. in alternate days in 12 administrations (total amount: 36×10^6 I.U.). In these cases, an MRI with gadolinium has been performed at the end of the treatment, and two samples of the tumor fluid (about 1.5–2.0 mL) were acquired for each patient: the first one before starting the treatment and the second one just after the end of the treatment (24th day). In the remaining three patients, the fluid was obtained at the time of the surgical procedure. The volume of the total fluid contained in the cyst was measured by MRI Cavalieri method: [volume: Σ (area cyst slices)×distance between two slices in cm] [17].

The fluid samples were stored at −80°C until analyzed.

Reagents and instruments

All common chemicals and reagents were of analytical grade and were purchased from Farmitalia-Carlo Erba, (Milan, Italy), Merck (Damstadt, Germany) and Sigma Aldrich (St. Louis, MI, USA). Standards of α-defensins 1 and 2 were purchased from Bachem (Bubendorf, Swizterland).

The HPLC-ESI-MS apparatus was a ThermoFinnigan (San Jose, CA, USA) Surveyor HPLC connected by a T splitter to a diode-array detector and to an LCQ Deca XP Plus ion-trap mass spectrometer. The mass spectrometer was equipped with an ESI source. The chromatographic column was a Vydac (Hesperia, CA, USA) C8 column, with 5 μm particle diameter (column dimensions 150×2.1 mm).

Table 1 Summary of clinical data of patients, cyst volumes, and α-defensins nanomoles

N	Age (years)	Surgical procedure	Intracystic treatment	Cyst volume (cm^3)		α-Defensin1[a] (nanomoles)		α-Defensin2[a] (nanomoles)		α-Defensin3[a] (nanomoles)		Total α-defensins[a] (nanomoles)	
				Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	3	Endoscopy	Yes	65.4	0.9	41.7	0.1	44.6	0.2	14.1	0.0	100.4	0.4
2	6	Craniotomy	Yes	15.6	2.5	21.2	2.4	49.6	6.5	21.6	2.5	92.5	11.5
3	3	Endoscopy	Yes	57.9	2.5	64.4	4.5	97.9	14.2	26.3	1.7	188.7	20.5
4	4	Endoscopy	No	11.5		1.9		12.8		2.2		17.1	
5	6	Endoscopy	No	44.6		72.7		131.9		59.1		263.8	
6	5	Endoscopy	No	17.1		2.8		15.4		1.0		19.3	

[a] Nanomoles of α-defensins in the cyst (percentage error less than 5%)

Reversed-phase high-performance liquid chromatography coupled to ion-trap mass spectrometry

The cystic fluid was diluted by 0.1% aqueous 2,2,2-trifluoroacetic acid (TFA) in a 1:1 (*v*/*v*) ratio. Solution was centrifuged at 8,000×*g* for 10 min in order to discharge precipitating proteins and treated in a 1:1 ratio (*v*/*v*) with $CHCl_3$ to remove lipids. After separation of the organic phase, 100 μL of the aqueous solution, corresponding to 50 μL of the cystic fluid, were analyzed by HPLC ESI-MS. For the HPLC separation, the following eluents were utilized: (eluent A) 0.056% aqueous TFA and (eluent B) 0.050% TFA in acetonitrile–water 80/20 (*v*/*v*). The gradient applied was linear from 0% to 100% of eluent B in 55 min, at a flow rate of 0.30 mL/min. At the end of the column, a T splitter addressed a flow rate of 0.20 mL/min towards the diode array detector and 0.10 mL/min towards the mass spectrometer. The diode array detector was set at a wavelength of 214 and 276 nm. The eluent was not directed toward the electrospray source for the first 5 min of separation, in order to avoid damage to the ion trap-mass spectrometer deriving from elevated concentration of electrolytes (salts, small polar molecules). The ESI source spray voltage was 4.50 kV and the capillary temperature 220°C. Mass spectra were collected every 3 ms in the positive ion mode.

Determination of α-defensins nanomoles

The nanomoles of α-defensins in cystic fluid were determined by the eXtracted Ion Current (XIC) peak area revealed by ion-trap mass spectrometer. The following *m*/*z* values were chosen to reveal the peak of each defensin: α-defensin 1 (861.5±0.5 (tetra charged); 1,148.4±0.5 (three charged); 1,722.0±0.5 (bi-charged)), α-defensin 2 (843.7±0.5 tetra charged); 1,124.6±0.5 (three-charged); 1,686.5±0.5 (bi-charged), α-defensin 3 (872.6±0.5 tetra charged); 1,163.1±0.5 (three charged); 1,744.1±0.5 (bi-charged). By using standards of α-defensins, it was established that XIC peak area is linearly related to the picomoles of α-defensins 1–3 according to the relationship (XIC area)/picomole= 8.9×10^5 (R=0.991). Nanomoles of α-defensins contained in the cyst were established considering the ratio between the analyzed volume (either 25 or 50 μL) and the cyst volume measured as described in the previous section.

Results

The HPLC-ESI-MS analysis of the acidic soluble fraction of cystic fluid before IFN-α treatment revealed, among other uncharacterized peaks, a peak eluting at 18.4 min. At the end of the chromatographic profile, relevant quantities of plasma proteins, mainly serum albumin, were also detected. Deconvolution of the averaged ESI spectra, carried out by Bioworks Browser or by MagTran 1.0 software [30], revealed three peptides with average masses (M_{av}) of 3,441.9±0.5; 3,370.9±0.5; and 3,486.2±0.5 Da. Figure 1 shows an example of this detection. The experimental M_{av} corresponded, within the experimental error, to the theoretical M_{av} of α-defensin 1 (3,442.1 Da; three disulfide bridges; Swiss Prot Code P59665; http://us.expasy.org/tools), α-defensin 2 (3,371.0 Da; three disulfide bridges; P59665/6), and α-defensin 3 (3,486.1 Da; three disulfide bridges; P59666), respectively. Identification was confirmed using standard of α-defensins as previous described [23].

Nanomoles of defensins, determined as described in the Method section, are reported in Table 1, together with cyst volume determined in the six patients.

Intracystic treatment with INF-α (three out of six patients) resulted efficient in all cases, with the almost complete disappearance of the tumor cyst at the end of the treatment and at a mean of 18 months follow-up (Fig. 2). In the remaining three cases, INF-α treatment has not been performed because of: (1) the occurrence of one technical failure related to the endoscopic positioning of the catheter; (2) one brain edema related to the leakage of IFN-α; and (3) one too pasty cystic fluid to be pulled off. All these patients underwent the surgical resection of the craniopharyngioma.

Discussion

The cystic portion of the craniopharyngioma is filled with secreted fluid, cholesterol crystals, and epithelial cells. This portion of the tumor is associated to a major risk of recurrence in spite of the presence of benign histological features, thus suggesting a proliferative mechanism in its formation and growth. However, a very few investigations were devoted to the identification of the components present in the cyst of craniopharyngioma [2, 10, 12, 22, 25, 27, 28].

In the present study, we examined the fluid content of six cystic craniopharyngiomas in not-previously operated children. Three patients underwent a successful intra-tumoral treatment with INF-α, showing the almost complete disappearance of the tumor cyst at the end of the therapy. Such an encouraging result was previously observed in other patient submitted to the same treatment [5].

HPLC-ESI MS allowed to characterize α-defensins 1–3 as relevant components of cyst fluids and to follow their decrease as a function of INF-α treatment. The presence of these antimicrobial peptides could suggest a possible involvement of the innate immune response in the formation and maintenance of the craniopharyngioma-associated cyst or simply be expression of an inflammatory

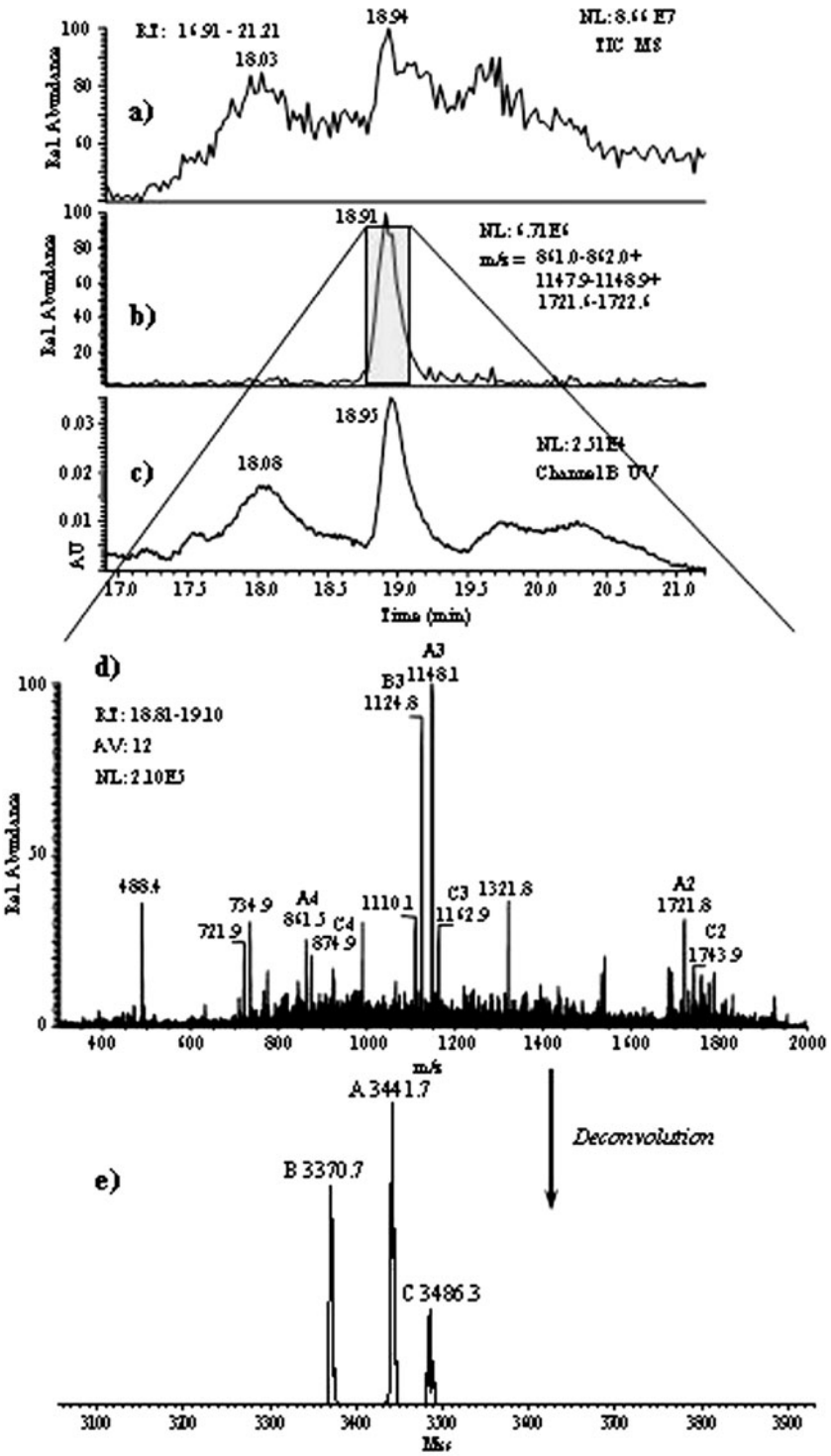

Fig. 1 HPLC-ESI-MS detection of α-defensins 1–3 in the acidic soluble fraction of cyst fluid of craniopharyngioma. **a** Total ion current (TIC) profile recorded by the ion-trap MS apparatus in the 16.91–21.21 min elution range. **b** Example of the extracted ion current (XIC) strategy applied to the detection of α-defensins 1 using *m*/*z* values 861.5±0.5 (tetra-charged), 1,148.4±0.5 (three-charged), 1,722.0±0.5 (bi-charged). **c** UV profile (276 nm) in the 16.91–21.21 min range. **d** ESI spectrum in the 18.81–19.10 min range (average of 12 spectra), whom deconvolution (**e**) provided the mass of α-defensin 1 (3,441.7 Da), α-defensin 2 (3,370.7 Da) and α-defensin 3 (3,496.3 Da)

response taking place within the craniopharyngioma cyst. The last phenomenon could be suggested by studies indicating a possible roleplayed in the cyst formation by the increased porocity of the neovascularity associated with the tumor [10, 29]. Human α-defensins 1–3, indeed, constitute 30–50% of the total protein content of neutrophil azurophil granules [7, 8], with a well-known powerful antibacterial and antiviral activity. The α-defensin expression is significantly increased in saliva of patients with oral squamous cell carcinomas [16], in the fluid of jaw cysts [19], and in the plasma of patients with sepsis and meningitis [21].

The pathology of cyst formation has been always debated [10, 22]. More recent studies suggest that the

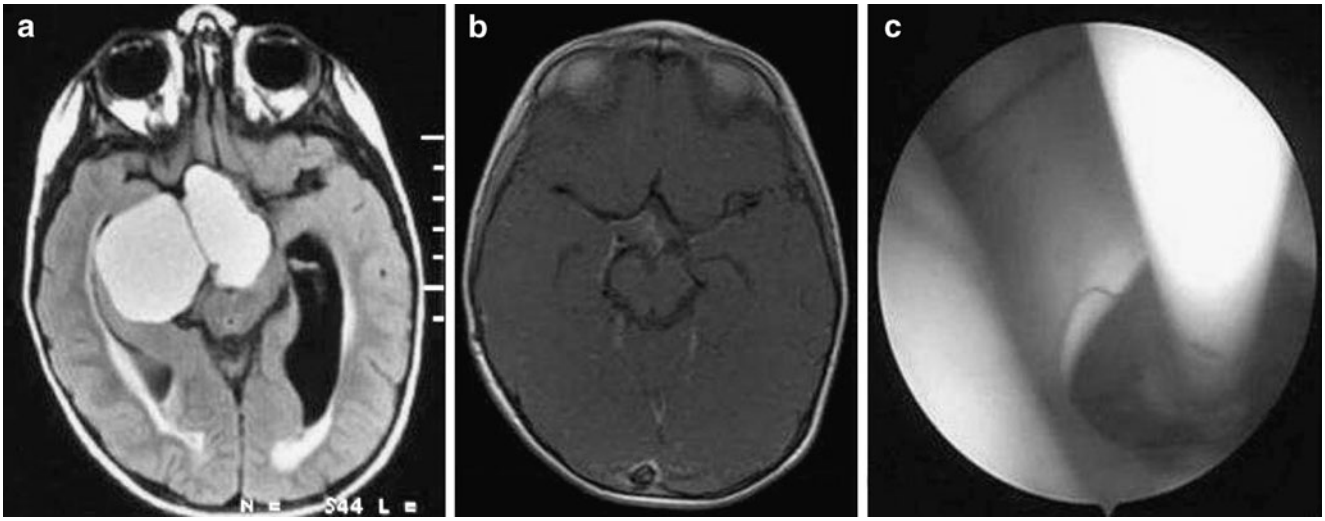

Fig. 2 **a** The pre-operative MRI shows the supra-sellar cystic craniopharyngioma. **b** The MRI performed at the end of the intratumoral treatment shows the complete resolution of the cystic component of the craniopharyngioma. **c** Intraoperative positioning of the intracystic catheter

cystic component of craniopharyngioma could be also the result of an active production of the fluid by the epithelial cells of tumor [2, 11, 27]. Indeed, the cyst fluid recurs after multiple aspirations and the epithelium of the cyst is the actively proliferating component due to the presence of secretory squamous cells and of zymogen granules [2, 11, 27]. The detection of high level α-defensins in the cyst fluid of craniopharyngiomas seems to exclude anorigin from disruption of the blood–brain barrier, because the concentration of defensins is very low in serum [7]. An alternative explanation could be the involvement of the innate immune response which would account for some specific features of the cystic fluid such as its inflammatory properties responsible for the chemical meningitis sustained by craniopharyngioma fluid spill into the subarachnoid space [24], the vasospasm induced by the contact of the cyst fluid to arteries [14], evidence of local synthesis of IgG and elevated lactate levels in cyst fluid, which do not correlate with serum lactate levels [2], and finally, the strong affinity of natural killer (NK) cells to craniopharyngioma [20, 25].

The most recent experience on intracystic therapy of craniopharyngioma is based on the use of INF-α. This chemotherapic agent is usually given for the treatment of squamous cells carcinoma. A recent study on the use of INF-α against craniopharyngioma suggests a possible role of the Fas-induced apoptosis [12]. INF-α belongs to a family of proteins with antiproliferative and immunomodulatory functions but it is used as antitumoral agent in many malignant diseases such as leukemias and solid tumors [26]. This chemotherapic may have direct and indirect effects on tumoral cells. The direct effects include the cytotoxicity, induction of a cellular differentiation, and a partial inhibition on the neoplastic proliferation. The indirect effects comprehend the stimulation of different immunological functions (with the involvement of T lymphocytes, NK cells, and macrophages), the inhibition of neoplastic vascolarization, the production of cytokines that interfere with the tumoral growth factors and its cell adhesion molecules [6]. Furthermore, these indirect effects are particularly remarkable in the squamous cell carcinomas, in which INF-α and retinoic acid represents good therapeutic candidates [15]. On these grounds, Cavalheiro et al. [4] has recently proposed to utilize INF-α for the treatment of cystic craniopharyngioma. These authors justify their successful clinical experience of the reduction in size of the craniopharyngioma to the activation of the apoptotic pathway FasL mediated [12]. However, the mechanisms of action of INF-α is still under debate [6]: the use of INF-α in clinical oncology is indeed generally based to exploit the anti-proliferative, pro-apoptotic and anti-angiogenetic activities, rather than the recently described effects on immune cells [6]. The reduction of α-defensins, observed in this study, could derive from the direct antitumoral effect on the squamous epithelial cells of the craniopharyngioma cyst, which reduces their secretory activity, from its immuno-modulatory effects on the recruitment of cells of the innate immune systems, from its anti-angiogenetic activity or a combination of the above mentioned mechanisms. In any case and interestingly, the clinical good outcome observed in our three patients correlates with the gradual reduction of α-defensins 1–3 amounts. More cases are necessary to confirm such relationship and, on the contrary, the absence of such correlation in subjects whose cystic tumor does not respond to the interferon-α therapy. Even more interesting results could be obtained by evaluating the level of the defensins 1–3 in the tumor parenchyma directly.

Conflicts of interest The authors declare they have no financial and personal interests in the material discussed in the present paper.

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