EXPERIMENTAL STUDY

Differential effects of natural flavonoids on growth and iodide content in a human Na^+/I^- symporter-transfected follicular thyroid carcinoma cell line

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Abstract

Objective: Natural flavonoids (plant pigments) have been shown to inhibit thyroid peroxidase (TPO) *in vitro* and the growth of thyroid cancer cell lines. We have studied the role of flavonoids on the iodide transport and the growth of the human follicular thyroid cancer cell line (FTC133) which was stably transfected with the human Na^+/I^- symporter (hNIS).

Design and methods: Cells were treated with flavonoids (0.5–50 μM) for 0, 2, 4 and 6 days; ^{125}I content and ^{125}I efflux of the cells and DNA content were measured.

Results: Cell growth was inhibited significantly at day 6 by most flavonoids. Eight out of ten flavonoids decreased the ^{125}I content of the cells at day 4. Morin did not influence the ^{125}I content of the cells and, surprisingly, myricetin increased the ^{125}I content of the cells. Kaempferol, apigenin, luteolin and F21388 decreased NIS mRNA expression after 15, 29 and 48 h; after 96 h NIS mRNA returned to normal.

Conclusion: As TPO is not present in this cell line, the effects of the flavonoids on the iodide uptake are not related to organification. Myricetin was the only flavonoid studied that increased the influx and decreased the efflux of iodide. The effect of myricetin (decreased growth and increased retention of iodide) can be of therapeutic value in the radioiodide treatment of thyroid carcinoma.

European Journal of Endocrinology 150 557–564

Introduction

Differentiated thyroid carcinoma has an overall favorable prognosis due to its biological behavior and the highly effective primary therapy consisting of total thyroidectomy and radioiodide therapy. The uptake of iodide is an essential step in thyroid hormone synthesis and essential for the treatment of thyroid carcinoma with radioiodide. This transport process is mediated by the Na^+/I^- symporter (NIS). Rat NIS was cloned in 1996, followed in the same year by human (h)NIS (1–4). Alterations in NIS mRNA expression and NIS function are present in various thyroid disorders: iodide deficiency, congenital hypothyroidism, autoimmunity and thyroid carcinoma (5–11). In many patients with recurrent or metastatic thyroid carcinoma, radioiodide uptake by metastases is insufficient. In this situation, no effective curative therapies are currently available. Therefore the search for compounds that have both cytotoxic and beneficial effects on radioiodide uptake is of major importance. In this respect, the flavonoids are an interesting class of substances.

Natural flavonoids are plant pigments that are found in our daily diet, such as all edible plants, nuts, grain

and fruit. They are hydroxylated polyphenols and can be classified into different categories: flavonols (quercetin, kaempferol, morin, myricetin), flavonones (naringenin), flavones (luteolin, apigenin) and isoflavones (genistein) (12).

There is evidence that some of the flavonoids can inhibit growth or induce apoptosis of tumor cells, including thyroid cancer cells (13–16) *in vitro*. Different mechanisms are involved (13): they inhibit enzymes, such as protein tyrosine kinase and DNA topoisomerase I and II which are crucial for cellular proliferation (13, 14).

Apart from inhibiting cell proliferation, flavonoids have effects on thyroid function and thyroid hormone metabolism *in vitro* and *in vivo*. Some of the natural flavonoids inhibit thyroid peroxidase (TPO) *in vitro* (12) and in humans (17). The synthetic flavonoid F21388 (3-methyl-4',6-hydroxy-3',5'-dibromoflavone), developed as a thyroid hormone (T_4) analog, displaces T_4 from transthyretin (TTR), and inhibits 5'-deiodinase activity *in vitro* (18–20). *In vivo*, F21388 causes tissue-specific changes in thyroid hormone metabolism in the rat (21–25).

Thus, although flavonoids may have beneficial properties for the treatment of thyroid carcinoma by

inhibiting thyroid cancer growth, it is important to know whether this effect is compromised by negative effects of flavonoids on iodide kinetics, given the documented effects of flavonoids on iodide organification in thyroid follicles. We chose to investigate the effects of flavonoids on follicular thyroid carcinoma using a human follicular thyroid cancer cell line, FTC133. This cell line expresses most proteins present in differentiated thyroid tissue (thyrotropin receptor (TSH-R), thyroglobulin (Tg), thyroid transcription factors TTF-1, PAX-8) in contrast to most poorly differentiated cell lines available, but does not express TPO and hNIS, comparable with many thyroid cancers. However, there are reports that FTC133 cells express lowered levels of thyroid-specific proteins (26).

In this study we have investigated the effects of several flavonoids on the growth and ^{125}I content in the follicular thyroid carcinoma cell line FTC133, which is stably transfected with the hNIS (27, 28).

Materials and methods

The follicular thyroid carcinoma cell line FTC133 (donated by Drs Goretzki and Simon, University of Düsseldorf, Germany) was stably transfected with hNIS (donated by Dr Jhiang, Ohio State University, OH, USA) and named FTC133-NIS30 as described earlier (27, 28). The empty vector cell-transfected cell line FTC133-V4 was also used in the experiments. Cells were grown in Dulbecco's modified Eagle's medium and modified Ham's-F12 medium (1:1) supplemented with 10% fetal calf serum and penicillin/streptomycin. The cells were treated with flavonoids in a dose range from 0.5 to 50 μM for 0, 2, 4 and 6 days. The flavonoids used were: myricetin, kaempferol, apigenin, luteolin, morin, quercetin, naringenin, genistein and fisetin (Table 1) (Sigma/Aldrich, Aldrich Chemie BV, Zwijndrecht, The Netherlands) and the synthetic flavonoid F21388 (a gift from K Irmscher, Merck, Darmstadt, Germany). Flavonoids (10 mM stock) were dissolved in 10 μl dimethylsulphoxide and 400 μl ethanol. In control conditions, cells were grown with the addition of the solvent only. All experiments were performed in 12-well plates. On each plate, one row

of controls was included to correct for minor differences in plate conditions. For that reason, results are expressed as a percentage of the control. The value of the control cells was defined as 100% at each time point (e.g. days 2, 4, 6 or time in 0–120 min). This is specified in the Figure legends. Medium with/without flavonoids was refreshed every other day.

Effects of flavonoids on cell growth

Effects of flavonoids on cell growth of FTC133-NIS30 and the empty vector-transfected cell line FTC133-V4 were measured on days 0, 1, 2, 4 and 6, using a quantitative fluorescent DNA assay. Dose-dependent effects were studied on day 4. Flavonoid concentrations ranged from 0.5 to 50 μM .

Effects of flavonoids on iodide content of the cells

Acute effects of flavonoids on ^{125}I content ^{125}I content was measured as previously described (27, 28). In brief, cells were cultured in 12-well plates and, 24 h before the addition of Na^{125}I , the medium was changed to serum-free (27). For the ^{125}I content measurements, cells were washed with Hank's balanced salt solution (HBSS) and incubated with 500 μl HBSS containing 0.2 μCi Na^{125}I and 1 nM NaI at 37 °C. Flavonoids (5 and 50 μM) were added to the incubation fluid together with the radioiodide. Radioiodide content of the cells was measured after 5, 10, 20, 40 and 60 min. Cells were washed twice with ice-cold HBSS, and the ^{125}I content (result from ^{125}I uptake, ^{125}I efflux and ^{125}I re-uptake) of the cells was extracted with ethanol/HCl. ^{125}I content was calculated as percentage of the dose of Na^{125}I and expressed as % of control cells at each time point.

Long-term effects of flavonoids

^{125}I content Cells were treated with flavonoids (50 μM) for 2, 4 and 6 days ^{125}I content was measured as described above. The dose-dependent effects of flavonoids on ^{125}I content were studied on day 4. Flavonoid concentrations ranged from 0.5 to 50 μM .

Iodide efflux Cells were cultured for 4 days with flavonoids (50 μM) and were incubated for 30 min with HBSS containing 1 nM NaI and 0.2 μCi Na^{125}I . Thereafter, the cells were washed every 15 min by adding 500 μl HBSS for 2 h. Radioactivity in all fluid fractions was counted.

RT-PCR

After 15, 28, 49 and 96 h of flavonoid treatment, total RNA from the cell lines was isolated and reverse transcribed. The cDNA was used as a template for PCR

Table 1 Flavonoids used in the experiments.

Myricetin	3,3',4',5,5',7-hexahydroxyflavone ($\text{C}_{15}\text{H}_{10}\text{O}_8$)
Quercetin	3,3',4',5,6-pentahydroxyflavone ($\text{C}_{15}\text{H}_{10}\text{O}_7$)
Fisetin	3,3',4',7-tetrahydroxyflavone ($\text{C}_{15}\text{H}_{10}\text{O}_6$)
Naringenin	4',5,7-trihydroxyflavone ($\text{C}_{15}\text{H}_{12}\text{O}_5$)
Morin	2',3,4',5,7-pentahydroxyflavone ($\text{C}_{15}\text{H}_{10}\text{O}_7$)
Luteolin	3',4',5,7-tetrahydroxyflavone ($\text{C}_{15}\text{H}_{10}\text{O}_6$)
Apigenin	4',5,7-trihydroxyflavone ($\text{C}_{15}\text{H}_{10}\text{O}_5$)
Genistein	4',5,7-trihydroxyisoflavone ($\text{C}_{15}\text{H}_{10}\text{O}_5$)
Kaempferol	3,4',5,7-tetrahydroxyflavone ($\text{C}_{15}\text{H}_{10}\text{O}_6$)
F21388	3-methyl-4',6-hydroxy-3',5'-dibromoflavone

amplification. The internal standard QB2 plasmid (donated by Dr Shire, Sanofi Elf Bio Recherches, Labège, France) was used to standardize cDNA for β_2 microglobulin expression by competitive PCR as described previously (29, 30). The sequences of the hNIS PCR primers were used as reported (2). Primer sequences for TTF-1, PAX-8, Tg, TSH-R and TPO were designed using primer III design software (<http://www.genome.wi.mit.edu>) and checked against GenBank to avoid cross-reactivity with other known sequences. Amplicon lengths for hNIS, TTF-1, PAX-8, Tg, pendrin and TPO were 415, 393, 399, 401, 400 and 298 bp respectively. Sequences are available upon request to the authors. Primers were manufactured by Eurogentec, Seraing, Belgium. PCR conditions were as follows: cDNA was denatured at 95 °C for 5 min, followed by 28–38 cycles of 95 °C for 20 s, 56 °C for 60 s and 72 °C for 30 s and extension at 72 °C for 3 min.

Statistics

All experiments were performed four to six times. Data are expressed as means $\pm$ S.E.M. or S.D. as indicated in the legends. Within-group differences were analyzed using the paired *t*-test. Between-group differences were analyzed with an unpaired *t*-test. SPSS-10 was used for calculations.

A value of $P < 0.05$ was considered to be significant.

Results

Growth

Growth was assessed by measuring the amount of DNA per well. Table 2 shows the DNA values ($\mu\text{g}/\text{well}$) of FTC133-NIS30 cells and FTC133-V4 cells without flavonoids (controls) after 1, 2, 4 and 6 days. To compare results, data are expressed as percentage of control in Fig. 1. Growth was inhibited time dependently by most of the flavonoids. Fifty micrograms of luteolin, genistein and F21388 inhibited significant growth by day 2. At day 4, myricetin inhibited growth significantly and at day 6 growth was inhibited by 50 μM morin, apigenin and kaempferol. Naringenin, quercetin and fisetin did not influence growth significantly. Growth was also inhibited by the same flavonoids in FTC133-V4 (data not shown).

Table 2 Growth of FTC133-NIS30 and FTC133-V4 after 1, 2, 4 and 6 days without flavonoids, expressed as μg DNA/well. Values are the means $\pm$ S.D. ($n = 12$ per time-point).

Day	FTC133-NIS30 ($\mu\text{g}/\text{well}$)	FTC133-V4 ($\mu\text{g}/\text{well}$)
1	9.6 $\pm$ 0.3	11.4 $\pm$ 0.2
2	13.6 $\pm$ 0.9	14.2 $\pm$ 0.6
4	22.9 $\pm$ 2.1	22.2 $\pm$ 1.8
6	33.8 $\pm$ 1.7	32.7 $\pm$ 0.9

Acute effects of flavonoids

Table 3 shows the ^{125}I content of FTC133-NIS30 without flavonoids expressed as a percentage of the total activity of ^{125}I -Na/well after 5, 10, 20, 40 and 60 min. The effects of the acute addition of 5 μM flavonoids is depicted in Fig. 2A; only kaempferol decreased the ^{125}I content significantly at all time points. F21388 decreased ^{125}I content significantly from 10 min onward. Myricetin increased the ^{125}I content during the first 10 min, followed by a decrease to control values afterwards. At 50 μM , all flavonoids except myricetin decreased ^{125}I content at 5 and 10 min (Fig. 2B). In contrast, myricetin significantly increased ^{125}I content at all time points. DNA content was not influenced during the incubation period from 5 to 60 min (data not shown).

Figure 1 Effects of 50 μM flavonoids on the growth of the cell line FTC133-NIS30 measured by a quantitative fluorescence DNA assay and expressed as percentage of control (O). Cells were grown in the presence of flavonoids for 2, 4 and 6 days. The value of control cells was defined as 100% at each time point (days 0, 2, 4 and 6). (A) Kaempferol (Δ), genistein ($\square$), luteolin ($\diamond$) and F21388 ($\blacktriangledown$) decreased the amount of DNA. (B) Myricetin ($\star$), apigenin ($\times$) and morin ($\blacktriangle$) decreased the amount of DNA. Values are means $\pm$ S.E.M. ($n = 6$). *at least $P < 0.05$, **significant for two flavonoids, ***significant for three flavonoids. Fisetin, naringenin and quercetin did not affect the growth significantly.

Table 3 Examples of ^{125}I content of FTC133-NIS30 without addition of flavonoids after incubation for 5, 10, 20, 40 and 60 min with ^{125}I -Na, expressed as percentage of the total activity of ^{125}I /well. Values are the means $\pm$ S.D. ($n = 12$ per time-point).

Time (min)	^{125}I content (% activity ^{125}I /well)
5	5.7 $\pm$ 0.2
10	8.6 $\pm$ 0.4
20	16.3 $\pm$ 0.5
40	20.1 $\pm$ 1.1
60	22.2 $\pm$ 0.7

Long-term effects of flavonoids

After 4 days of incubation with flavonoids, most of them decreased the ^{125}I content 30 min after the addition of ^{125}I -Na. Morin did not influence the ^{125}I content and only myricetin showed an increase (Fig. 3). The decrease in ^{125}I content was not really

Figure 2 Flavonoids (A) 5 μM or (B) 50 μM were added to the incubation fluid. Iodide uptake was measured using 0.2 μCi ^{125}I Na and 1 nM NaI in HBSS, after 5, 10, 20, 40 and 60 min. The ^{125}I content is corrected for μg DNA expressed as percentage of control. The value of control cells was defined as 100% at each time-point (0, 5, 10, 20, 40 and 60 min). Only kaempferol (Δ) and F21388 (∇) decreased the ^{125}I content at all time-points. Apigenin and luteolin did not have a significant effect. At 50 μM myricetin ($\star$) increased the ^{125}I content. Apigenin ($\times$), luteolin ($\diamond$), F21388 (∇) and kaempferol (Δ) decreased the ^{125}I content. Values are means $\pm$ S.E.M. ($n = 4$). *at least $P < 0.05$, **significant for two flavonoids.

Figure 3 Long-term effects of 50 μM flavonoids on ^{125}I content *in vitro*. Cells were grown for 4 days in the presence of flavonoids. ^{125}I content was measured using 0.2 μCi ^{125}I Na and 1 nM NaI in HBSS for 30 min. The ^{125}I content is corrected for μg DNA and expressed as percentage of control. The value of control cells was defined as 100%. Morin (M) did not affect the iodide uptake. Quercetin (Q), naringenin (N), apigenin (A), kaempferol (K), luteolin (L), genistein (G), fisetin (F) and F21388 (F2) decreased the ^{125}I content significantly. In contrast, myricetin (My) increased the ^{125}I content. Values are means $\pm$ S.E.M. ($n = 4$ per experiment). * $P < 0.05$.

dose dependent (Fig. 4). Interestingly, the increase of 35% in ^{125}I content was observed for myricetin already with 5 μM . Higher concentrations of myricetin did not increase the ^{125}I content further. At days 4 and 6, the effects of flavonoids on ^{125}I content were similar to those at day 2 (Fig. 5). As the FTC133-V4 cells do not express NIS, the iodide uptake with or without flavonoids was negligible.

Iodide efflux

After 4 days of incubation with 50 μM flavonoids, iodide efflux was measured. Figure 6A shows a faster efflux by apigenin, kaempferol, luteolin and F21388.

Figure 4 Effects of different concentrations of flavonoids on ^{125}I content *in vitro*. Cells were grown for 4 days in the presence of 0–50 μM flavonoids. ^{125}I content was measured using 0.2 μCi ^{125}I Na and 1 nM NaI in HBSS for 30 min. The ^{125}I content was measured per μg DNA, and expressed as percentage of control (O). The value of control cells was defined as 100% at each concentration. Values are the means $\pm$ S.E.M. ($n = 4$ per experiment). * $P < 0.05$. Luteolin ($\diamond$), F21388 (∇), fisetin ($\bullet$) and myricetin ($\star$).

Figure 5 The ^{125}I content in cells after 2, 4 and 6 days of incubation with flavonoids. Values are expressed per μg DNA, and as percentage of control (O). The value of control cells was defined as 100% at each time point (days 2, 4 and 6). Myricetin (★) increased ^{125}I content whereas apigenin (×), luteolin (◇) and kaempferol (△) decreased ^{125}I content. F21388 (▼) was used to compare synthetic flavonoids with natural flavonoids. The concentration of flavonoids used was $50\text{ }\mu\text{M}$. Values are means $\pm$ S.E.M. ($n = 4$ per experiment). *at least $P < 0.01$, ****significant for four flavonoids.

In contrast, myricetin lowered the efflux, leading to a higher retention of ^{125}I (Fig. 6B).

RNA expression

Figure 7 shows NIS mRNA expression at different time-points after the addition of flavonoids. Kaempferol, apigenin, luteolin and F21388 decreased NIS mRNA expression significantly after 15, 29, 46 and 49 h of treatment. After 96 h, NIS mRNA expression was again comparable with control, with the exception of kaempferol. Myricetin did not change NIS mRNA expression. The other flavonoids also did not influence NIS expression (data not shown). FTC133-V4 did not express hNIS and was used as a negative control.

Flavonoids did not influence the mRNA expression of pendrin, Tg, PAX-8, TTF-1, and TSH-R (data not shown). TPO was never present (data not shown).

Discussion

The purpose of this study was to investigate whether flavonoids affect growth of follicular thyroid cancer cells and what the effects are on iodide accumulation, apart from the documented effects of flavonoids on iodide organification in thyroid follicles (17). We found that most flavonoids inhibit growth as well as ^{125}I content. However, apart from inhibiting growth, myricetin significantly increased iodide influx.

The effects of flavonoids on the follicular thyroid carcinoma cell line FTC133 as observed in this study are in line with the earlier observations on growth in non-thyroid (14) and thyroid carcinoma cell lines (15, 16). As growth (defined as increased DNA content) is the result of proliferation on one side and necrosis/apoptosis on the other side, the effects of flavonoids

Figure 6 Effects of flavonoids on efflux and retention of iodide. Cells were grown for 4 days in the presence of $50\text{ }\mu\text{M}$ flavonoids. After adding $0.2\text{ }\mu\text{Ci Na}^{125}\text{I}$ and 1 nM NaI in HBSS for 30 min cells were washed every 15 min with HBSS containing 1 pM NaI up to 2 h. (A) ^{125}I content in cells compared with control (O); apigenin (×), luteolin (◇) kaempferol (△) and F21388 (▼) increased the efflux. (B) Only myricetin (★) decreased the efflux of iodide, leading to an increased retention time of iodide. Only the flavonoids that exhibited significant effects are shown. Values are means $\pm$ S.E.M. ($n = 4$ per experiment). * $P < 0.05$, ****significant for four flavonoids.

can be attributed to one or more of these aspects. Although the mechanism is not clear, flavonoids may inhibit growth by interference in DNA synthesis and inducing apoptosis (13).

In this study, we have shown for the first time that natural flavonoids affect the *in vitro* ^{125}I content in a stably hNIS-transfected follicular thyroid carcinoma cell line. It had been shown that NIS mRNA and NIS protein expression in FTC133 cells had been induced by retinoic acid, but an increase in functional iodide uptake was not present (31). Most flavonoids decreased the ^{125}I content, whereas surprisingly myricetin increased ^{125}I content by 35% at $5\text{ }\mu\text{M}$. The effects on ^{125}I content are not related to the class of flavonoids, i.e. myricetin which increased the ^{125}I content, kaempferol which decreased the ^{125}I content and morin and quercetin which did not influence ^{125}I content all are flavonols. The flavones luteolin and apigenin behaved

Figure 7 An example is shown of the effects of some flavonoids on hNIS mRNA expression at different time-points. After 15, 28, 49 and 96 h of flavonoid treatment, total RNA from the cell lines was isolated and reverse transcribed. The cDNA was used as a template for PCR amplification. Amplicon length for hNIS was 415 bp. cDNA was denatured at 95 °C for 5 min, followed by 28 cycles of 95 °C for 20 s, 56 °C for 60 s and 72 °C for 30 s and extension at 72 °C for 3 min. H₂O and mRNA of FTC133-V4 were used as negative controls. Kaempferol, apigenin, luteolin and F21388 decreased this expression significantly after 15, 29, 49 and 96 h of treatment. After 96 h NIS mRNA expression is nearly normal, with the exception of kaempferol. Myricetin did not change NIS mRNA expression.

similarly to the synthetic flavonoid F21388. The structure of the latter is strikingly similar to luteolin (20). F21388 was included to compare results from earlier studies (21–24). This flavonoid was used as a model to study the interactions of flavonoids with the iodide–thyroid hormone system *in vivo* and *in vitro*. These differences in effects may be an indication of the

differences in the mechanisms of action; many mechanisms may be possible (13). This is also the case for the differences between acute effects and dose after 4 and up to 6 days of incubation. The absence of a clear dose-dependent effect is probably due to the presence of fetal calf serum in the medium during the incubation with the flavonoids. The maximum effect of myricetin is already obtained with 5 µM. F21388 and luteolin both have a stronger effect with 50 µM than with 20 µM. As both are known to bind TTR, it can be expected that the effect will be lower at the lower concentration, and that when saturation of the binding to TTR is obtained, resulting in higher free flavonoid concentrations, the effects will increase (19, 25).

The mechanism of action of the flavonoids on iodide uptake is not clear. In the steady state, iodide accumulation is the result of the influx mediated by NIS and of the efflux probably mediated by pendrin, the recently cloned apical iodide transporter, or channels such as the chloride channels (32). In our experiments, it appears that some flavonoids (kaempferol, apigenin, luteolin) decreased NIS mRNA expression at semi-quantitative PCR and from that it can be assumed that the influx will be affected. Other explanations, such as the interference of flavonoids with NIS mRNA half-life may be relevant. Non-genomic interference of flavonoids with NIS activity may also be of relevance as kaempferol inhibited iodide uptake as early as 5 min.

The efflux of iodide was increased by all flavonoids used, with the exception of myricetin. This may help to explain the decrease in iodide accumulation by these flavonoids as well. The increased ¹²⁵I content by myricetin appeared to be related to increased influx as well as a decreased efflux. In this latter aspect, the effects of flavonoids on pendrin may be relevant, but no effects on pendrin gene expression were observed. Other transporters are probably involved (32) or the post-translational effects of flavonoids on other proteins involved in the whole chain of iodide metabolism and/or direct effects due to physico-chemical interference with the function of these proteins can play a role. The effects of flavonoids on proteins and enzymes such as topoisomerases, Na⁺/K⁺-ATPases, heat shock proteins, cell cycle proteins and/or tyrosine kinases are well known (13).

The main focus on the activity of flavonoids on the thyroid in the literature was directed towards their effect (inhibition) on TPO (1). The cell line used in our studies is devoid of TPO, so the actions of the flavonoids seen here are independent of the organification-directed action of TPO, but only to the process of influx and efflux of inorganic iodide.

We have concluded that the growth of the follicular carcinoma cell line FTC133 and also ¹²⁵I content was decreased by most of the flavonoids. Only myricetin revealed additional beneficial effects on ¹²⁵I content. The increased iodide content and

decreased efflux, leading to a threefold increased retention of ^{125}I makes myricetin an interesting candidate for more efficient therapy of thyroid carcinoma. Further studies are necessary to test this hypothesis and to elucidate the underlying mechanisms of the action.

Acknowledgements

Some parts of these studies were presented in a preliminary form at the Annual Meeting of the European Thyroid Association, Warsaw, Poland in 2001 and Göteborg, Sweden in 2002.

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Received 30 September 2003

Accepted 11 December 2003