Endocrine, *vol. 18, no. 3, 303–308, August 2002* 0969–711X/02/18:303–308/$20.00

Combined Effect of Xenoestrogens and Growth Factors in Two Estrogen-Responsive Cell Lines

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It is now well recognized that estrogenic signaling mechanisms are far more complex than once thought. Several crosstalks between the estrogen receptor and other signaling pathways may influence the estrogenic stimulation of cell growth. Thus, the estrogenic effects of several environmental contaminants, now suspected to act as endocrine disrupters, may be influenced by a simultaneous stimulation of other signaling pathways. The aim of this study was to investigate whether the growth response of two estrogen-responsive cell lines, MCF-7 and GH3, treated with xenoestrogens might be affected by the addition of growth factors to their culture medium. Cells were treated with two known xenoestrogens, endosulfan and chlordane, alone or in the presence of insulin-like growth factor-1 and epidermal growth factor, respectively, and their growth was measured using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide proliferation assay. Our results show that treatment with endosulfan or chlordane as well as treatment with growth factors increased cell growth, while the administration of xenoestrogens together with growth factors triggered a partly additive response with no antagonist or synergistic effect. These results sustain a role for xenoestrogens in cellular growth.

Key Words: Xenoestrogens; estrogens; chlordane; endosulfan; insulin-like growth factor-1; epidermal growth factor.

Introduction

Several pollutants accumulated in the environment over the years are now suspected to have estrogenic activity on wildlife and human populations. Although these chemicals may affect adult populations, environmental evidence suggests that such estrogenic activity might be especially detrimental during embryonic development or early life *(1–3)*. For example, interference by endocrine-disrupting chemicals or growth factors on normal signaling pathways has been the proposed cause of certain disorders of the reproductive system *(4–6)*, but there is no strong evidence yet to support this hypothesis.

The mechanisms by which environmental pollutants can trigger cellular responses comparable to those of estrogens are still largely unknown. Recent reports have suggested that, in addition to the direct binding of these xenoestrogens to the estrogen receptor (ER) or to the estrogen-related receptor *(7)*, several other non-genomic mechanisms might also be involved. Such mechanisms include the agonist-independent activation of the ER through tyrosine phosphorylation as well as activation of other second messengers in response to stimulation with both 17β-estradiol (E_2) and growth factors *(8,9)*. Furthermore, the still poorly understood activity of a rapid, membrane-bound subpopulation of ER has been shown to interact with second-messenger cascades *(5, 10)*. Given the multiplicity of interactions suspected to occur between these pathways, estrogens and growth factors are therefore very likely to influence their respective actions. Indeed, crosstalk between estrogens and growth factor signaling pathways has been demonstrated in animals *(11)* as well as in cell lines *(12–16)*.

To verify whether xenoestrogens and growth factor signaling pathways could influence each other, we decided to test for possible interactions between two xenoestrogens and two growth factors in cells known to respond to estrogen and growth factor stimulation separately. Endosulfan is known to have estrogenic activity even if it binds poorly to the ER *(17)*; it increases the transcription in various reporter gene assays *(18)* and stimulates MCF-7 cell growth *(19)*. On the other hand, the estrogenic potential of chlordane is somewhat less clear. It apparently does not bind the ER, and reports suggest that it has no effect on cell growth *(19)*; however, it can activate transcription in gene reporter assays *(20)* and has an effect on the sexual development of laboratory animals *(21)*. Chlordane is also among the 12 persistent organic pollutants for which the United Nations Environment Program has requested a complete ban.

Human breast tumor MCF-7 and rat pituitary GH3 cell lines are known to respond to estrogens by increasing their growth rate *(19,22)* and have often been used to test the

Received May 21, 2002; Revised July 26, 2002; Accepted July 26, 2002.
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estrogenic potential of suspected xenoestrogens *(23–25)*. Insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) are known to induce growth in MCF-7 and GH3, respectively *(12,26,27)*. Using a metabolic proliferation assay, we determined whether the addition of growth factors to known endocrine disrupters may have a compounding or disrupting effect on the growth of MCF-7 and GH3 cell lines. Our results show that the combined action of chlordane or endosulfan together with IGF-1 or EGF triggered a response with no synergistic or antagonistic effects. The increase in growth we observed when xenoestrogens and growth factors were used together was partially additive.

Results

Stimulation of MCF-7 Growth by E_2, Chlordane, Endosulfan, or IGF-1 Alone

To establish the optimal conditions for the stimulation of MCF-7 cells with xenoestrogens or a growth factor known to stimulate their growth, we first determined the concentration of E_2, chlordane, endosulfan, or IGF-1 capable of inducing the maximal cell growth separately. Our preliminary experiments showed that the maximal growth stimulation of MCF-7 cells was reached at 10^{-9}, 10^{-7}, and 10^{-7} *M* for E_2, endosulfan, and chlordane, respectively (data not shown). We then measured MCF-7 cell growth after 2, 3, 4, and 6 d (Fig. 1A) to establish the optimal duration of estrogenic stimulation. The maximal increase in growth was attained with administration of E_2 after 2 d (101.8% over control). However, no significant difference was observed between 2- and 3-d treatments with E_2 (Fig. 1A). Administration of endosulfan or chlordane gave similar results, with a maximal increase after 2 and 3 d, respectively (Fig. 1A). Again, statistical analysis did not reveal significant differences between 2- and 3-d treatments with endosulfan or chlordane. We then established the optimal conditions for IGF-1 stimulation by subjecting MCF-7 cells to increasing concentrations of IGF-1 for 1, 2, or 3 d (Fig. 1B). The maximal growth stimulation, 40.0% over control, was achieved with a dose of 25 ng/mL of IGF-1 after a period of 24 h (Fig. 1B). In all subsequent experiments, MCF-7 cells were exposed to E_2, chlordane, or endosulfan for 3 d with a supplement of 25 ng/mL of IGF-1 for the last 24 h of incubation.

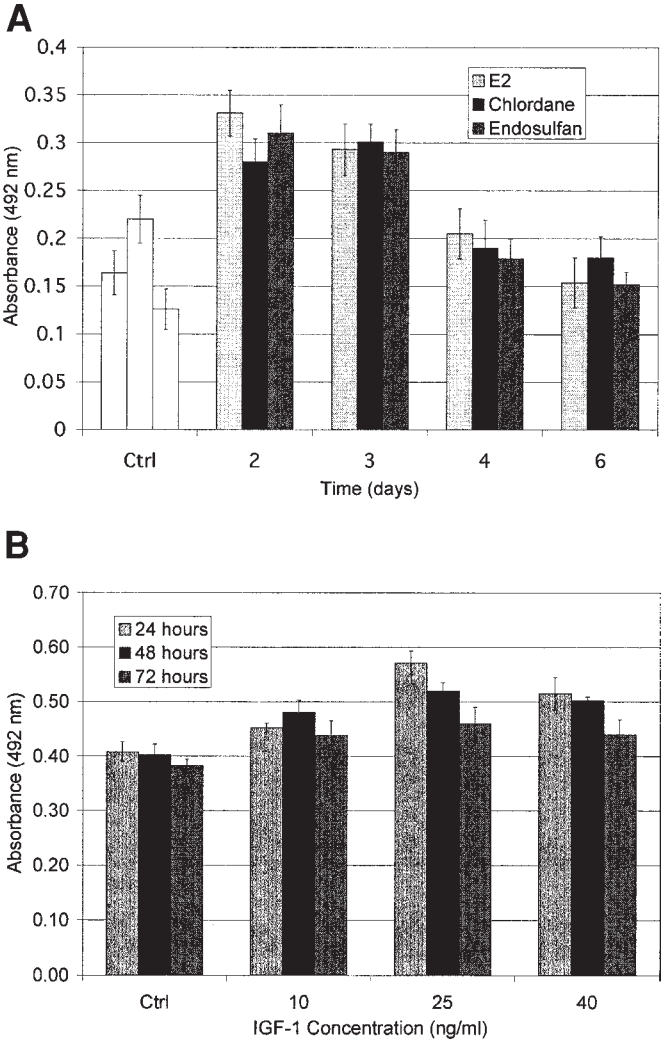

Fig. 1. Stimulation of MCF-7 growth by E_2, xenoestrogens, or IGF-1. MCF-7 cells were grown for a total of 6 d and received no treatment (controls) or were treated for the last 2, 3, 4, and 6 d with either 10^{-9} *M* E_2, 10^{-7} *M* chlordane, or 10^{-7} *M* endosulfan (**A**). The maximal induction of MCF-7 cell growth was obtained with 2 d of treatment for E_2 and endosulfan and 3 d for chlordane, with no significant differences between 2- and 3-d treatments. (**B**) MCF-7 cells were also stimulated with progressive doses of IGF-1 and allowed to grow for 24, 48, or 72 h. The maximal growth stimulation, 40.0% over control, was achieved with a dose of 25 ng/mL of IGF-1 after a period of 24 h.

Stimulation of GH3 Cell Growth by E_2, Chlordane, Endosulfan, or EGF Alone

GH3 cells were treated for 1, 2, 3, and 4 d with 10^{-9} *M* E_2, 10^{-7} *M* endosulfan, or 10^{-7} *M* chlordane (Fig. 2A). The maximal induction of GH3 growth was obtained after 1 d for E_2, 3 d for endosulfan, and 2 d for chlordane; however, there were no significant differences among 1-, 2-, and 3-d treatments (Fig. 2A). GH3 cell cultures were then stimulated with increasing doses of EGF and allowed to grow for 1, 2, or 3 d (Fig. 2B). The maximal growth stimulation was achieved with a dose of 25 ng/mL of EGF for a period of 2 d, with no significant difference between 2- and 3-d treatments. In all subsequent experiments, GH3 cells were exposed to E_2, chlordane, or endosulfan for 3 d with a supplement of 25 ng/mL of EGF for the whole period.

Combined Effects of IGF-1 and E_2, Chlordane, or Endosulfan in MCF-7 Cells

To test for a possible interaction between IGF-1 and xenoestrogen stimulation of MCF-7 cells, cultures were grown with increasing doses of endosulfan or chlordane as well as with 10^{-9} E_2 as internal reference for 3 d with or without the addition of 25 ng/mL of IGF-1 for the last 24 h (Fig. 3A,B).

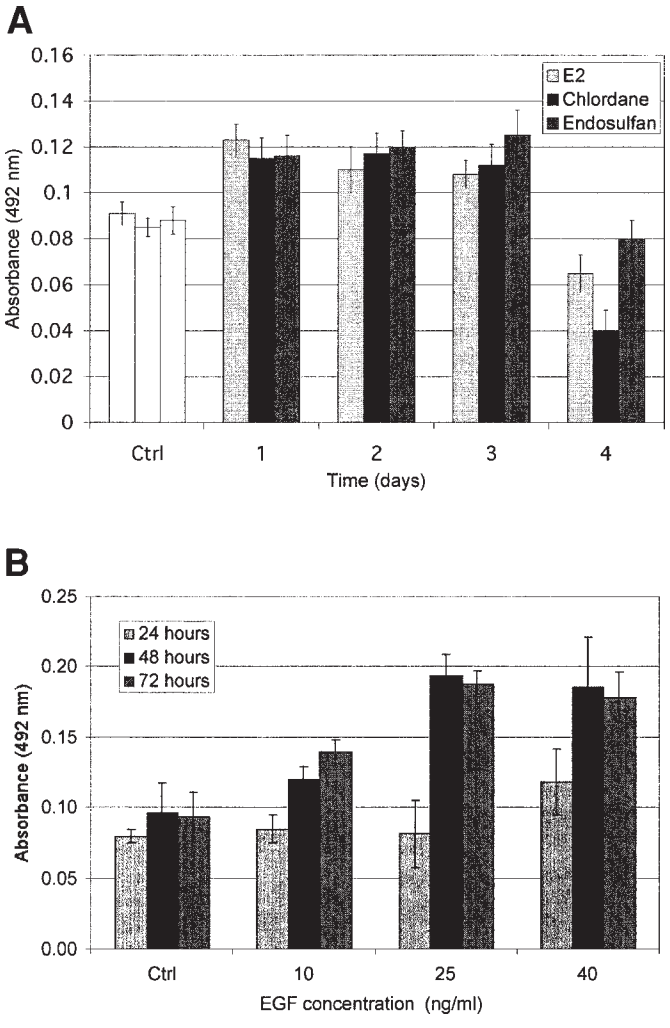

Fig. 2. Stimulation of GH3 cell growth by E_2, xenoestrogens, or EGF. GH3 cells were grown for a total of 4 d and received no treatment (controls) or were treated for the last 1, 2, 3, and 4 d with either 10^{-9} *M* E_2, 10^{-7} *M* chlordane, or 10^{-7} *M* endosulfan (**A**). The maximal induction of GH3 cell growth was obtained at 1 d with E_2, 2 or 3 d with chlordane, and 3 d with endosulfan. However, there were no significant differences among 1-, 2-, and 3-d treatments. (**B**) GH3 cells were also stimulated with progressive doses of EGF and allowed to grow for 24, 48, or 72 h. The maximal growth stimulation, 111% over control, was achieved with a dose of 25 ng/mL of IGF-1 after a period of 48 h, with no significant difference after 72 h.

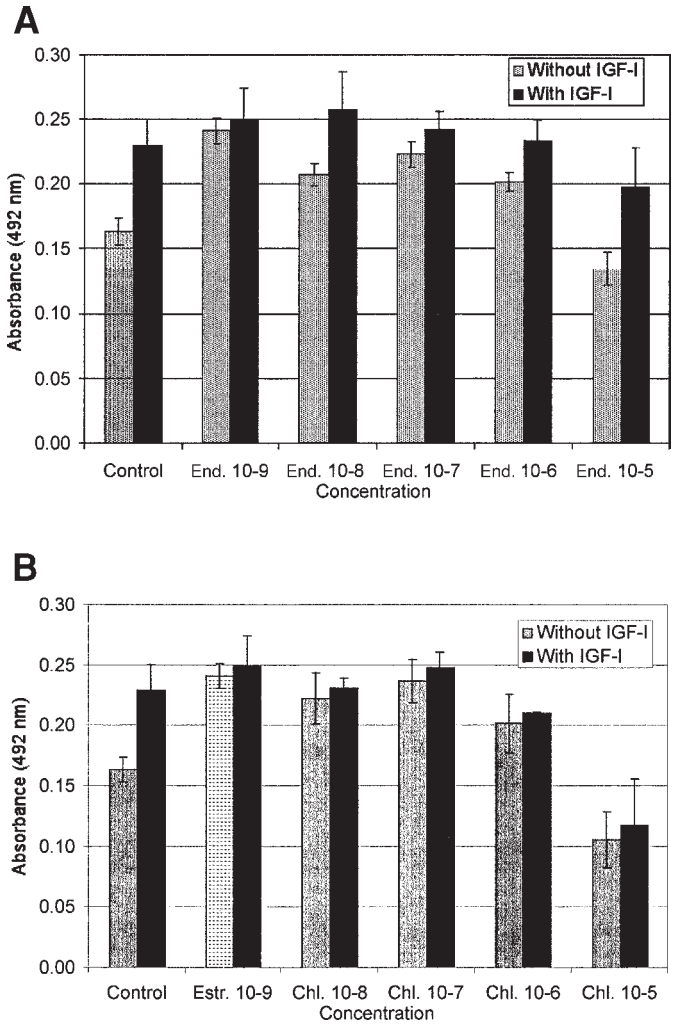

Fig. 3. Combined effects of IGF-1 and chlordane or endosulfan on MCF-7 cells. MCF-7 cells received no treatment (control) or were grown in increasing doses of either endosulfan (**A**) or chlordane (**B**) for 2 d before being stimulated with 25 ng/mL of IGF-1 for a further 24 h, as described in Materials and Methods. (A) Endosulfan maximally increased the growth of MCF-7 cells at a concentration of 10^{-7} *M* (36.8% over control), and costimulation with IGF-1 only slightly increased endosulfan stimulation. (B) Chlordane also increased the growth of MCF-7 cells (45.4% over the unstimulated control) at a concentration of 10^{-7} *M*, but the addition of IGF-1 did not significantly increase cell growth.

As shown in Fig. 3A, 10^{-8}, 10^{-7}, and 10^{-6} *M* endosulfan significantly increased the growth of MCF-7 cells. The maximal increase was observed for 10^{-7} *M* (36.8% over unstimulated control), and the addition of IGF-1 further increased cell growth, (49.1% over unstimulated control) (Table 1). Figure 3B shows that chlordane significantly increased the growth of MCF-7 cells at a concentrations of 10^{-7} and 10^{-8} *M*. Specifically, 10^{-7} *M* chlordane induced a maximal increase in MCF-7 cells (45.4% over unstimulated control) (Table 1). However, the addition of 25 ng/mL of IGF-1 did not show any statistical effect on chlordane stimulation of MCF-7 cells.

Because of the apoptotic appearance of the cultures (our unpublished observation), the significant decrease in cell growth observed with concentrations of endosulfan or chlordane at 10^{-5} *M* was attributed to cell death. As summarized in Table 1, the addition of 10^{-9} *M* E_2, 10^{-7} *M* endosulfan, or 10^{-7} *M* chlordane for 3 d significantly increased MCF-7 cell growth.

Combined Effects of EGF and E_2, Chlordane, or Endosulfan in GH3 Cells

A possible interaction between xenoestrogens and EGF signaling pathways in GH3 cells was also tested with increas-

Table 1
Maximal Growth Increase
in MCF-7 Cells After Growth Factor Stimulation[a]

	–IGF-1	+IFG-1	Δ	*p* Value
Control	0.0	40.5	40.5	*
E_2 (10^{-9} *M*)	47.9	53.4	5.5	
Endosulfan (10^{-9} *M*)	36.8	49.1	12.3	*
Chlordane (10^{-9} *M*)	45.4	52.2	6.8	

[a] The variation in growth (Δ) between cultures treated without (–) or with (+) growth factors was calculated for the xenoestrogen treatments. IGF-1 increased growth of MCF-7 cells when they were treated with either E_2, endosulfan, or chlordane. A significant difference is observed for control cells and cells treated with endosulfan.

ing doses of endosulfan or chlordane as well as 10^{-9} *M* E_2 as internal reference for 3 d in the presence or absence of 25 ng/mL of EGF (Fig. 4A,B). Endosulfan maximally increased the growth of GH3 cells at a concentration of 10^{-9} *M* (84.5% over unstimulated control) (Table 2), and the addition of 25 ng/mL of EGF further stimulated the cell growth up to 132.4% over the unstimulated control (Fig. 4, Table 1). However, all concentrations of endosulfan significantly increased GH3 cellular growth (Fig. 4A). The addition of EGF produced an additive stimulation (131.0% over unstimulated control) (Table 2). Moreover, statistical analysis revealed that GH3 cell growth stimulated with either endosulfan or chlordane did not significantly differ (Table 2). As summarized in Table 2, the addition of either E_2, endosulfan, or chlordane as well as EGF increased cell growth. The addition of E_2 and the xenoestrogens together with EGF further increased growth by a smaller margin than either stimulation separately.

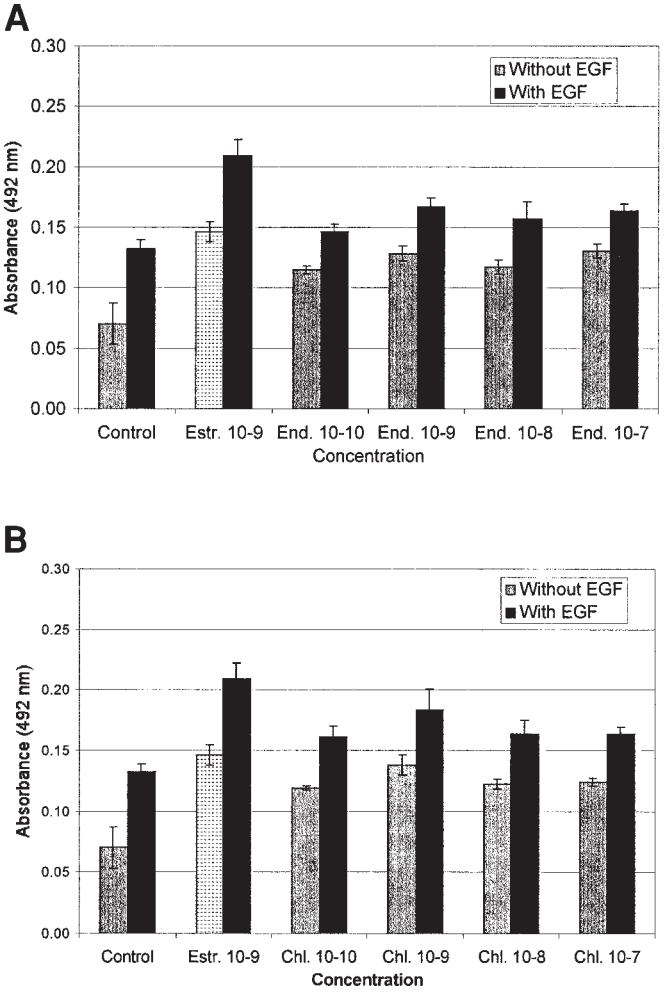

Fig. 4. Combined effects of EGF and chlordane or endosulfan on GH3 cells. GH3 cells received no treatment (control) or were grown in increasing doses of either endosulfan (**A**) or chlordane (**B**) before being stimulated with 25 ng/mL of EGF for 72 h, as described in Materials and Methods. (A) Endosulfan maximally increased the growth of GH3 cells at a concentration of 10^{-9} *M*. The addition of EGF produced an additive stimulation of cell growth (63.7% over the unstimulated control). (B) Chlordane also maximally increased the growth of GH3 cells at a concentration of 10^{-9} *M*. Again, the addition of EGF further increased cell growth (82.4% of unstimulated control).

Discussion

The aim of our study was to verify whether the response of two cell lines to the concurrent stimulation of xenoestrogens and growth factors could show any crosstalk between their signaling pathways. It has previously been shown that growth factors and E_2 can influence their respective signaling pathways via crosstalk *(13,22,28,29)*, but there still is no evidence that xenoestrogens such as endosulfan or chlordane may influence growth factor signaling pathways nor be influenced by them. Steroid hormones and peptide growth factors are known to play an important role in the growth and development of sex-related characteristics such as masculinization, feminization, hermaphroditism, altered sexual behavior, as well as decreased reproductive success in wildlife species *(30–32)*. We investigated whether xenoestrogens and growth factor signaling pathways may influence each other and thus suggest an explanation for the apparently enhanced sensibility of developing organisms to xenoestrogens.

Our results show that E_2, endosulfan, chlordane, or growth factors alone can significantly increase the growth of MCF-7 or GH3 cells. We also observed a partial additive effect when xenoestrogens and growth factors were combined in GH3 cells. However, we did not observe any significant synergism or disrupting effects on cell growth when treatments with xenoestrogens and growth factors were combined. In MCF-7 cells, the addition of IGF-1 during treatments with E_2, endosulfan, or chlordane only marginally increased growth. Chlordane, endosulfan, as well as IGF-1 increased cell growth by a similar margin. It is, however, possible that cell growth reached a plateau that could not be surpassed with the combined stimulation of xenoestrogens and IGF-1.

Table 2
Maximal Growth Increase
in GH3 Cells After Growth Factor Stimulation[a]

	–EGF	+EFG	Δ	*p* Value
Control	0.0	87.3	87.3	*
E_2 (10^{-9} *M*)	107.0	195.8	88.7	*
Endosulfan (10^{-9} *M*)	84.5	132.4	47.9	*
Chlordane (10^{-9} *M*)	95.8	159.2	63.4	*

[a]EGF increased cell growth of GH3 in control cells as well as in cells treated with E_2, endosulfan, or chlordane. The maximal effect on cell proliferation was observed with the administration of E_2. The significance (*) of the growth increase after growth factor stimulation was assessed at $p < 0.05$. All results represent the mean of four replicates ± SEM. Data are expressed as percentage of control.

Conversely, with the GH3 cell line, cellular growth increased when xenoestrogens and EGF were combined. It would seem that, contrary to MCF-7 cells, neither xenoestrogens nor growth factors alone induce maximal growth of GH3 cells, and that costimulation can indeed increase growth to a higher level.

In our experiments, E_2 reached its maximal stimulation after only 1 d and slightly decreased thereafter. By contrast, endosulfan or chlordane induced a maximal stimulation after 2 or 3 d, suggesting a slower or delayed mechanism of action compared to E_2, as previously reported *(23)*. Since treatment with xenoestrogens combined with growth factors did not produce a synergistic or disrupting effect on cell growth, our results moderate the hypothesis suggesting that their respective signaling pathways can influence each other. Note, however, that our results cannot rule out balanced effects of each pathway and that increased growth is only one of the responses of MCF-7 and GH3 cells to estrogenic stimulation. We therefore cannot rule out the possibility that crosstalk occurs at other cellular levels, such as on the regulation of particular genes *(14,23)*.

Our study indicates that there can be at least an additive effect of both xenoestrogens and growth factor stimulation. Thus, the presence of growth factors in studies examining the effects of xenoestrogens, in vitro as well as in vivo, may affect the xenoestrogenic response and should therefore be taken into account in developing organisms.

Materials and Methods

Experimental Design

Two cell lines, MCF-7 and GH3, known for their increased growth after stimulation with either estrogens or growth factors were used in parallel experiments to determine whether the combined effect of both stimulations could show a disruptive or synergistic effect of either signaling pathway. In a first series of experiments, the optimal conditions (time and concentration) to obtain maximal growth with endosulfan, chlordane, and growth factors were determined separately. Finally, the growth of MCF-7 and GH3 was then measured after treatments with endosulfan or chlordane together with growth factors and IGF-1 or EGF, respectively.

Cell Culture

Two cell lines known to respond to estrogens and to growth factors (MCF-7 and GH3) were obtained from American Type Culture Collection. For all assays, 2,000 (MCF-7) or 15,000 (GH3) cells were seeded in 96-well plates and allowed to attach for 2 d in minimum essential medium (MEM) without phenol red + 10% fetal bovine serum (FBS) for MCF-7 cells, or Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) without phenol red + 12.5% horse serum + 2.5% FBS for GH3 cells. Growth media were then replaced with MEM without phenol red + 1% FBS without steroids for MCF-7 cells and with DMEM/F-12 + 1% FBS without steroids for GH3 cells. Cultures were allowed to grow for 2 days in 1% serum without steroids before the addition of E_2, endosulfan (hexachloro-norbornene-dimethanol cyclic sulfite), chlordane (octachloro-hexahydro-methano-indene), or the vehicle alone (dimethylsulfoxide [DMSO]) for the indicated period of time. All sera used were rendered steroid free beforehand with activated charcoal *(33)*.

Xenoestrogens and Growth Factors

To test for their potential effect on MCF-7 and GH3 cell growth, increasing concentrations of either endosulfan or chlordane (Chem Service) diluted in DMSO were added to the media. The final concentration of DMSO was 0.1% in all samples. Cells were also treated with E_2 (Sigma, St. Louis, MO), as internal control. To test the effects of growth factors, MCF-7 cells were stimulated with the indicated concentration of IGF-1 (Sigma) for the last 24 h of culture, whereas GH3 cells were treated with EGF (Sigma) at the indicated concentrations for the entire 3 d of treatment *(12,26,27)*.

Growth Measurement

Cell growth was inferred by the measurement of total metabolic activity using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT) test *(34)*. XTT (purchased from Sigma) is a soluble tetrazolium salt that is converted into formazan by cell dehydrogenases. Absorbance is measured directly in 96-well plates at 492 nm (with a reference wavelenght of 690 nm) 2 h after the addition of XTT to cell culture medium at a concentration of 1 mg/mL in the presence of 1% phenazine methosulfate as a coupling agent.

Statistical Analysis

Statistical analysis was performed according to the Wilcoxon Mann-Whitney nonparametric test *(35)* using the SPSS 6.1 program. Significance (*) was assessed at $p < 0.05$ for each treatment. All results represent the mean of four replicates ± SEM.

Acknowledgments

We are grateful to Dr. Sonya Grenier for help with the statistical analysis. We also thank Drs. Claude Gicquaud and Hélène Glemet for critical review of the manuscript. This study was supported by National Sciences and Engineering Research Council of Canada (NSERC) grants no. 170571 and 21906.

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